

**REMARKS**

The claims have been amended to clarify the invention. In particular, claim 1 has been amended to recite an antigenic epitope of SEQ ID NO:1 "from about amino acid residue G59 to about amino acid residue D75, and from about amino acid residue S455 to about amino acid residue T478 of SEQ ID NO:1", a biologically active portion of SEQ ID NO:1 "from about amino acid residue T32 to about amino acid residue L136 of SEQ ID NO:1" and a naturally occurring variant of SEQ ID NO:1 having at least 95% sequence identity to SEQ ID NO:1. Support for this amendment is found in the specification, for example, at p. 4, lines 3-5, at p. 9, lines 14-20, and at p. 11, lines 3-5. Claim 2 has been amended to delete the recitation of non-elected inventions and to recite a fragment of SEQ ID NO:2 consisting of SEQ ID NO:3. Claim 2 has also been amended to recite a naturally-occurring variant of SEQ ID NO:2 "having at least 85% identity to SEQ ID NO:2". Support for the amendment to SEQ ID NO:2 is found in the specification, for example, at p. 11, lines 8-17. No new matter is added by these amendments, and entry of the amendments is therefore requested.

**35 U.S.C. § 101, Rejection of Claims 1-6**

The Examiner has rejected claims 1-6 under 35 U.S.C. § 101, because the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility. The Examiner summarized claims 1-6 and stated that the disclosed utilities for the polynucleotide of SEQ ID NO:2 encoding a STEAP-related protein of SEQ ID NO:1 include diagnosis and treatment of cancer, particularly prostate hyperplasia and prostate cancer, production and screening of antibodies that specifically bind SEQ ID NO:1. The Examiner stated that neither the specification nor any art of record teaches what SEQ ID NO:2 is, what it does do, do not teach a utility for any of the fragments or the derivatives claimed, do not teach a relationship to any specific diseases or establish any involvement in the etiology of any specific diseases.

The Examiner stated that the asserted utility for SEQ ID NO:2 is based on the assertion that SEQ ID NO:1, encoded by SEQ ID NO:2, has chemical and structural homology to STEAP protein, a prostate specific marker, and that in particular, SEQ ID NO:1 and STEAP protein share 43% identity. The Examiner then summarized various structural and possible functional sequence motifs recited in SEQ ID NO:1 at pp. 10-11 of the specification, including the six predicted transmembrane domains shared by the two proteins, and further acknowledged that SEQ ID NO:2 is over-expressed in a prostate cancer cell line, LNCaP, as compared to PrEC non-tumorigenic prostate epithelial cells.

The Examiner stated, however, that the specification does not disclose any actual biological activity of SEQ ID NO:1, nor any data confirming that the portion from T32 to L136 of SEQ ID NO:1 has any biological activity. The Examiner then summarized the teachings of a number of articles alleged to support the Examiner's contention that the effects of dissimilarities between SEQ ID NO:1 and STEAP protein upon protein structure and function cannot be predicted. See Bowie et al (1990); Burgess et al. (1990); Lazar et al. (1988); Bork (2000); and Scott et al.(1999). The Examiner concluded that, given these teachings, with a 57% dissimilarity between SEQ ID NO:1 and the STEAP protein, the function of SEQ ID NO:1 could not be predicted.

#### Applicants Response

The Examiner is first of all reminded of the legal standard for utility as applied to patentable inventions. To meet the utility requirement of sections 101 and 112 of the Patent Act, the patent applicant need only show that the claimed invention is "practically useful," *Anderson v. Natta*, 480 F.2d 1392, 1397, 178 USPQ 458 (CCPA 1973) and confers a "specific benefit" on the public. *Brenner v. Manson*, 383 U.S. 519, 534-35, 148 USPQ 689 (1966). As discussed in a recent Court of Appeals for the Federal Circuit case, this threshold is not high:

An invention is "useful" under section 101 if it is capable of providing some identifiable benefit. See *Brenner v. Manson*, 383 U.S. 519, 534 [148 USPQ 689] (1966); *Brooktree Corp. v. Advanced Micro Devices, Inc.*, 977 F.2d 1555, 1571 [24 USPQ2d 1401] (Fed. Cir. 1992). ("to violate Section 101 the claimed device must be totally incapable of achieving a useful result"); *Fuller v. Berger*, 120 F. 274, 275 (7th Cir. 1903) (test for utility is whether invention "is incapable of serving any beneficial end"). *Juicy Whip Inc. v. Orange Bang Inc.*, 51 USPQ2d 1700 (Fed. Cir. 1999).

While an asserted utility must be described with specificity, the patent applicant need not demonstrate utility to a certainty. In *Stiftung v. Renishaw PLC*, 945 F.2d 1173, 1180, 20 USPQ2d 1094 (Fed. Cir. 1991), the United States Court of Appeals for the Federal Circuit explained:

An invention need not be the best or only way to accomplish a certain result, and it need only be useful to some extent and in certain applications: "[T]he fact that an invention has only limited utility and is only operable in certain applications is not grounds for finding lack of utility." *Envirotech Corp. v. Al George, Inc.*, 730 F.2d 753, 762, 221 USPQ 473, 480 (Fed. Cir. 1984).

The specificity requirement is not, therefore, an onerous one. If the asserted utility is described so that a person of ordinary skill in the art would understand how to use the claimed invention, it is sufficiently specific. See *Standard Oil Co. v. Montedison, S.p.a.*, 212 U.S.P.Q. 327, 343 (3d Cir. 1981). The specificity requirement is met unless the asserted utility amounts to a "nebulous expression"

such as “biological activity” or “biological properties” that does not convey meaningful information about the utility of what is being claimed. *Cross v. Iizuka*, 753 F.2d 1040, 1048 (Fed. Cir. 1985).

In addition to conferring a specific benefit on the public, the benefit must also be “substantial.” *Brenner*, 383 U.S. at 534. A “substantial” utility is a practical, “real-world” utility. *Nelson v. Bowler*, 626 F.2d 853, 856, 206 USPQ 881 (CCPA 1980).

If persons of ordinary skill in the art would understand that there is a “well-established” utility for the claimed invention, the threshold is met automatically and the applicant need not make any showing to demonstrate utility. Manual of Patent Examination Procedure at § 706.03(a). Only if there is no “well-established” utility for the claimed invention must the applicant demonstrate the practical benefits of the invention. *Id.*

Once the patent applicant identifies a specific utility, the claimed invention is presumed to possess it. *In re Cortright*, 165 F.3d 1353, 1357, 49 USPQ2d 1464 (Fed. Cir. 1999); *In re Brana*, 51 F.3d 1560, 1566; 34 USPQ2d 1436 (Fed. Cir. 1995). In that case, the Patent Office bears the burden of demonstrating that a person of ordinary skill in the art would reasonably doubt that the asserted utility could be achieved by the claimed invention. *Id.* To do so, the Patent Office must provide evidence or sound scientific reasoning. See *In re Langer*, 503 F.2d 1380, 1391-92, 183 USPQ 288 (CCPA 1974). If and only if the Patent Office makes such a showing, the burden shifts to the applicant to provide rebuttal evidence that would convince the person of ordinary skill that there is sufficient proof of utility. *Brana*, 51 F.3d at 1566. The applicant need only prove a “substantial likelihood” of utility; certainty is not required. *Brenner*, 383 U.S. at 532.

Knowledge of the biological function or role of a biological molecule has never been required to show real-world benefit. In its most recent explanation of its own utility guidelines, the PTO acknowledged so much (66 F.R. at 1095):

[T]he utility of a claimed DNA does not necessarily depend on the function of the encoded gene product. A claimed DNA may have specific and substantial utility because, *e.g.*, it hybridizes near a disease-associated gene or it has gene-regulating activity.

By implicitly requiring knowledge of biological function for any claimed nucleic acid, the Examiner has, contrary to law, elevated what is at most an evidentiary factor into an absolute requirement of utility. Rather than looking to the biological role or function of the claimed invention, the Examiner should have looked first to the benefits it is alleged to provide. The identification of SEQ ID NO:1 as a prostate-specific antigen related to STEAP is not based on any known biological function for the known STEAP

protein described by Hubert et al. (see IDS reference # 5) SEQ ID NO:1 was identified as a STEAP related protein by a combination of criteria; 1) sequence homology and structural features (e.g., transmembrane domains) shared between SEQ ID NO:1 and STEAP (Figure 2); 2) prostate-specific expression of SEQ ID NO:1 (Table 1); and 3) over-expression of SEQ ID NO:1 in prostate hyperplasia and prostate cancer (Table 2) and in a prostate tumor cell line modeling human prostate cancer (Table 3). All of these properties are shared by the STEAP protein described by Hubert et al.

With regard to the Examiner's allegation that the degree of sequence homology between SEQ ID NO:1 and STEAP protein is insufficient to confer a relationship between the two proteins, applicants reply that it is undisputed, and readily apparent from the patent application, that the polypeptide encoded for by the claimed polynucleotide shares more than 40% sequence identity over 490 amino acid residues with STEAP. Furthermore, the two proteins share the six transmembrane domains characteristic of a cell surface antigen such as STEAP. This is more than enough homology to demonstrate a reasonable probability that the utility of STEAP can be imputed to the claimed invention (through the polypeptide it encodes). It is well-known that the probability that two unrelated polypeptides share more than 40% sequence homology over 70 amino acid residues is exceedingly small. Brenner et al., Proc. Natl. Acad. Sci. 95:6073-78 (1998) (Exhibit A). Given homology in excess of 40% over many more than 70 amino acid residues, the probability that the polypeptide encoded for by the claimed polynucleotide is related to STEAP is, accordingly, very high.

While the Examiner has cited literature identifying some of the difficulties that may be involved in predicting protein function, none suggests that functional homology cannot be inferred by a reasonable probability in this case. Most important, none contradicts Brenner's basic rule that sequence homology in excess of 40% over 70 or more amino acid residues yields a high probability of functional homology as well. Nor do they contradict the evidence that the predicted transmembrane domains shared by the two proteins indicate the likelihood that SEQ ID NO:1, like STEAP, is a cell surface antigen. At most, these articles individually and together stand for the proposition that it is difficult to make predictions about function with certainty. The standard applicable in this case is not, however, proof to certainty, but rather proof to reasonable probability.

As evidence of the credibility of applicants prediction of SEQ ID NO:1 as a STEAP-related protein, the enclosed sequence alignment with GenBank sequence g15418732, designated STAMP1, (six-transmembrane protein of prostate-1) (Exhibit B) independently confirms the identity of SEQ ID NO:1 as a STEAP-related protein. A copy of the article describing the STAMP1 protein is also attached (Exhibit

C; Kormaz et al. (2002) J. Biol. Chem. 277:36689-36696 ) further confirming applicants predictions that SEQ ID NO:1 is a highly prostate-specific protein that is over-expressed in prostate cancer and therefore useful in the detection and diagnosis of prostate cancer.

In addition, however, applicants data reflecting the prostate-specific expression of the polynucleotide encoding SEQ ID NO:1 (Table 1) as well as its over-expression in prostate hyperplasia and prostate cancer (Table 2) and in a prostate cancer cell line modeling human prostate cancer (Table 3) confers a utility on the claimed polynucleotide that is independent of its relation to any other STEAP protein regardless of biological activity or function. Table 1 shows the predominant expression of the polynucleotide encoding SEQ ID NO:1 in male reproductive tissue (i.e., prostate tissue), and its over-expression in prostate hyperplasia and prostate cancer (Table 2). None of this evidence has been refuted by the Examiner. The Examiner has merely attempted to refute applicants data showing the over-expression of SEQ ID NO:2 in a prostate cancer cell line, LNCaP, relative to a non-tumorigenic cell line, PrEC, as not reflective of its potential use in the diagnosis of cancer; the Examiner cites various articles which she alleges shows the unpredictability of studies in cancer cell lines to any and all human cancers. See Drexler et al. (1993); Embleton et al. (1984); Hsu (1973); Freshney (1983); and Demer (1994).

None of the cited articles specifically address the well known use of prostate cell lines derived from human prostate cancers in modeling human prostate cancer. It is well known that human prostate cancer cell lines derived from human prostate cancer tissues, including LNCaP, are accepted models of human prostate cancer. Numerous articles have been published to that effect, and two such examples are attached as Exhibits D (Rothermund et al. (2002) Prostate Cancer and Prostatic Diseases 5:236-245) and E (Denmeade et al. (2003) The Prostate 54:249-257). Both of these articles specifically relate to the use of the LNCaP cell line in studying the expression of genes associated with prostate cancer. The JBC article cited above (Exhibit C) also describes the use of the LNCaP cell line in characterizing the STAMP1 protein as over-expressed in prostate cancer.

Indeed, if the use of cancerous cell lines were generally of such inconsequential value as the Examiner suggests, the evaluation of potential therapeutic agents for cancer treatment would require far more hazardous clinical studies at an earlier stage with a much higher risk for failure. Further, no such clinical studies are required for patentability to establish a "substantial likelihood" of a diagnostic or therapeutic use of an agent.

In summary, applicants submit that the prostate-specific expression of SEQ ID NO:2 and its differential expression in prostate hyperplasia and prostate cancer exemplified by data from both human

prostate tissues as well as a cell line derived from human prostate tissue, presents a substantial likelihood that the molecule would be useful in the detection and diagnosis of these diseases. Furthermore, the sequence similarity of SEQ ID NO:1 encoded by SEQ ID NO:2 with another prostate-specific protein, STEAP, that is similarly expressed further supports this likelihood and would be readily recognized by one of skill in the art at the time the application was filed. Withdrawal of the rejection of claims 1-6 under 35 U.S.C. § 101, is therefore requested.

35 U.S.C. § 112, First Paragraph, Rejection of Claims 1-6

The Examiner has rejected claims 1-6 under 35 U.S.C. § 112, first paragraph as containing subject matter which is not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The Examiner noted that a complement, as recited in claims 1 and 2, could be partial or complete, and that a cDNA "comprising" a fragment of SEQ ID NO:2 encompasses unrelated sequences that share with SEQ ID NO:2 a fragment of SEQ ID NO:2. The Examiner stated further that claims 1-6 read on polynucleotide variants of SEQ ID NO:2 wherein said variants have any type of substitution besides conservative substitution, at any amino acid throughout the length of the nucleic acid or peptide as well as insertions or deletions. Structural features, that could distinguish the claimed structural polynucleotide variants and nucleotide sequences encoding polypeptide variants from the nucleotide sequences known in the art, are missing from the disclosure. In addition, no common structural or functional attributes that identify the claimed polynucleotide variants are disclosed. The Examiner stated that since the disclosure fails to describe the common attributes or characteristics that identify members of the genus, and because the genus is highly variant, the disclosure is insufficient to describe the genus. The Examiner quoted *Vas-Cath v. Mahurkar* with respect to the principle that the invention, for the purposes of the written description inquiry, is *whatever is now claimed*. The Examiner also cited *The Regents of the University of California v. Eli Lilly* with respect to the courts holding that a generic statement which defines a genus of nucleic by *only* their functional activity does not provide adequate written description of the genus.

Applicants Response

Claim 1 has been amended to recite an antigenic epitope of SEQ ID NO:1, a biologically active portion of SEQ ID NO:1, and a variant of SEQ ID NO:1 having at least 95% identity to SEQ ID NO:1. Claim 2 has been amended to recite a fragment of SEQ ID NO:2 "consisting of" SEQ ID NO:3, and a variant of SEQ ID NO:2 having at least 85% identity to SEQ ID NO:2.

Applicants note that the definition of the "complement" of a cDNA at p. 6, lines 27-29 of the specification recites a cDNA "---which is completely complementary over its full length---", and therefore that the use of the term, as recited in the claims, explicitly excludes "partial" complementarity.

With respect to variants of the polypeptide sequence of SEQ ID NO:1 and SEQ ID NO:2, Applicants submit that the requirements necessary to fulfill the written description requirement of 35 U.S.C. 112, first paragraph, are well established by case law, some of which the Examiner has recited in support of her rejection under this statute:

. . . the applicant must also convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession of *the invention*. The invention is, for purposes of the "written description" inquiry, *whatever is now claimed*. *Vas-Cath, Inc. v. Mahurkar*, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991)

Attention is also drawn to the Patent and Trademark Office's own "Guidelines for Examination of Patent Applications Under the 35 U.S.C. Sec. 112, para. 1", published January 5, 2001, which provide that :

An applicant may also show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics<sup>42</sup> which provide evidence that applicant was in possession of the claimed invention,<sup>43</sup> i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure; or some combination of such characteristics.<sup>44</sup> What is conventional or well known to one of ordinary skill in the art need not be disclosed in detail.<sup>45</sup> If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate description requirement is met.<sup>46</sup>

Thus, the written description standard is fulfilled by both what is specifically disclosed and what is conventional or well known to one skilled in the art.

SEQ ID NO:1 and SEQ ID NO:2 are specifically disclosed in the application (see, for example, page 3, lines 5-9. Variants of SEQ ID NOs:1 and 2 are described, for example, at page 9, lines 14-20. Incyte clones in which the nucleic acids encoding the human STEAPRP were first identified and libraries from which those clones were isolated are described, for example, at page 9, lines 26-31 of the Specification. Chemical and structural features of STEAPRP are described, for example, on page 10, line 16 through p. 11, line 5. Given SEQ ID NOs:1 and 2, and the described chemical and structural features of SEQ ID NO:1, one of ordinary skill in the art would recognize naturally-occurring variants of SEQ ID NO:1 having 95% sequence identity to SEQ ID NO:1. Accordingly, the Specification provides an adequate written description of the recited polypeptide sequences.

**A. The Specification provides an adequate written description of the claimed “variants” of SEQ ID NO:1.**

The Office Action has further asserted that the claims are not supported by an adequate written description because

---the disclosure fails to describe the common attributes or characteristics that identify members of the genus, and because the genus is highly variant, the disclosure of specific nucleotide sequences and the ability to screen, is insufficient to describe the genus. (Page 15 of the Office Action).

**1. The present claims specifically define the claimed genus through the recitation of chemical structure**

Court cases in which “DNA claims” have been at issue commonly emphasize that the recitation of structural features or chemical or physical properties are important factors to consider in a written description analysis of such claims. For example, in *Fiers v. Revel*, 25 USPQ2d 1601, 1606 (Fed. Cir. 1993), the court stated that:

If a conception of a DNA requires a precise definition, such as by structure, formula, chemical name or physical properties, as we have held, then a description also requires that degree of specificity.

In a number of instances in which claims to DNA have been found invalid, the courts have noted that the claims attempted to define the claimed DNA in terms of functional characteristics without any reference to structural features. As set forth by the court in *University of California v. Eli Lilly and Co.*, 43 USPQ2d 1398, 1406 (Fed. Cir. 1997):

In claims to genetic material, however, a generic statement such as “vertebrate insulin cDNA” or “mammalian insulin cDNA,” without more, is not an adequate written description of the genus because it does not distinguish the claimed genus from others, except by function.

Thus, the mere recitation of functional characteristics of a DNA, without the definition of structural features, has been a common basis by which courts have found invalid claims to DNA. For example, in *Lilly*, 43 USPQ2d at 1407, the court found invalid for violation of the written description requirement the following claim of U.S. Patent No. 4,652,525:

1. A recombinant plasmid replicable in procaryotic host containing within its nucleotide sequence a subsequence having the structure of the reverse transcript of an mRNA of a vertebrate, which mRNA encodes insulin.



In *Fiers*, 25 USPQ2d at 1603, the parties were in an interference involving the following count:  
A DNA which consists essentially of a DNA which codes for a human fibroblast interferon-beta polypeptide.

Party Revel in the *Fiers* case argued that its foreign priority application contained an adequate written description of the DNA of the count because that application mentioned a potential method for isolating the DNA. The Revel priority application, however, did not have a description of any particular DNA structure corresponding to the DNA of the count. The court therefore found that the Revel priority application lacked an adequate written description of the subject matter of the count.

Thus, in *Lilly* and *Fiers*, nucleic acids were defined on the basis of functional characteristics and were found not to comply with the written description requirement of 35 U.S.C. §112; *i.e.*, “an mRNA of a vertebrate, which mRNA encodes insulin” in *Lilly*, and “DNA which codes for a human fibroblast interferon-beta polypeptide” in *Fiers*. In contrast to the situation in *Lilly* and *Fiers*, the claims at issue in the present application define polynucleotides or polypeptides in terms of chemical structure, rather than on functional characteristics. For example, the “variant language” of independent claim 1 recites chemical structure to define the claimed genus:

1. An isolated cDNA or the complement thereof comprising a nucleic acid sequence encoding:...b) a naturally occurring variant of SEQ ID NO:1 having at least 95% identity to the amino acid sequence of SEQ ID NO:1...

*check*

From the above it should be apparent that the claims of the subject application are fundamentally different from those found invalid in *Lilly* and *Fiers*. The subject matter of the present claims is defined in terms of the chemical structure of SEQ ID NO:1. In particular, the extensive chemical and structural features characterizing SEQ ID NO:1 and related STEAP proteins given at pp. 10-11 of the specification clearly encompass common structural attributes that identify the claimed polynucleotide variants, in opposition to the Examiner's contention. In the present case, there is no reliance merely on a description of functional characteristics of the polynucleotides or polypeptides recited by the claims. In fact, there is no recitation of functional characteristics. Moreover, if such functional recitations were included, it would add to the structural characterization of the recited polynucleotides or polypeptides. The polynucleotides or polypeptides defined in the claims of the present application recite structural features, and cases such as *Lilly* and *Fiers* stress that the recitation of structure is an important factor to consider in a written description analysis of claims of this type. By failing to base its written description inquiry “on whatever is now claimed,” the Office Action failed to provide an appropriate analysis of the present claims and how they differ from those found not to satisfy the written description requirement in *Lilly* and *Fiers*.

## 2. The present claims do not define a genus which is "highly variant"

Furthermore, the claims at issue do not describe a genus which could be characterized as "highly variant." Available evidence illustrates that the claimed genus is of narrow scope.

In support of this assertion, the Examiner's attention is again directed to by Brenner et al. (supra). Through exhaustive analysis of a data set of proteins with known structural and functional relationships and with <90% overall sequence identity, Brenner et al. have determined that 30% identity is a reliable threshold for establishing evolutionary homology between two sequences aligned over at least 150 residues. (Brenner et al., pages 6073 and 6076.) Furthermore, local identity is particularly important in this case for assessing the significance of the alignments, as Brenner et al. further report that ≥40% identity over at least 70 residues is reliable in signifying homology between proteins. (Brenner et al., page 6076.)

The present application is directed, *inter alia*, to STEAP proteins related to the amino acid sequence of SEQ ID NO:1. In accordance with Brenner et al, naturally occurring molecules may exist which could be characterized as STEAP proteins and which have as little as 40% identity over at least 70 residues to SEQ ID NO1. The "variant language" of the present claims recites, for example, polynucleotides encoding "a naturally-occurring amino acid sequence having at least 95% sequence identity to the sequence of SEQ ID NO:1" (note that SEQ ID NO:1 has 490 amino acid residues). This variation is far less than that of all potential STEAP proteins related to SEQ ID NO:1, i.e., those STEAP proteins having as little as 40% identity over at least 70 residues to SEQ ID NO:1.

## 3. The state of the art at the time of the present invention is further advanced than at the time of the *Lilly* and *Fiers* applications

In the *Lilly* case, claims of U.S. Patent No. 4,652,525 were found invalid for failing to comply with the written description requirement of 35 U.S.C. §112. The '525 patent claimed the benefit of priority of two applications, Application Serial No. 801,343 filed May 27, 1977, and Application Serial No. 805,023 filed June 9, 1977. In the *Fiers* case, party Revel claimed the benefit of priority of an Israeli application filed on November 21, 1979. Thus, the written description inquiry in those case was based on the state of the art at essentially at the "dark ages" of recombinant DNA technology.

The present application has a filing date of March 2001. Much has happened in the development of recombinant DNA technology in the 20 or more years from the time of filing of the applications involved in *Lilly* and *Fiers* and the present application. For example, the technique of polymerase chain reaction (PCR) was invented. Highly efficient cloning and DNA sequencing technology has been developed. Large databases of protein and nucleotide sequences have been compiled. Much of the raw material of the human and other genomes has been sequenced. With these remarkable advances one of

skill in the art would recognize that, given the sequence information of SEQ ID NO:1 and SEQ ID NO:2, and the additional extensive detail provided by the subject application, the present inventors were in possession of the claimed polynucleotide variants at the time of filing of this application. Withdrawal of the rejection of claims 1-6 under 35 U.S.C. § 112, first paragraph for lack of written description is therefore requested.

35 U.S.C. § 112, First Paragraph, Enablement Rejection of Claims 1-6

The Examiner has rejected claims 1-6 under 35 U.S.C. § 112, first paragraph, specifically, since the claimed invention is not supported by a well established utility for the reasons set forth in the rejection under 35 U.S.C. § 101 above, one skilled in the art clearly would not know how to use the claimed invention.

For the reasons set forth above in response to the rejection of these claims under 35 U.S.C. § 101, the claimed invention is supported by both a well established and a specific and substantial asserted utility and therefore that one skilled in the art would know how to use the claimed invention. Withdrawal of the rejection of claims 1-6 under 35 U.S.C. § 112, first paragraph, is therefore requested.

35 U.S.C. § 112, First Paragraph, Scope, Rejection of Claims 1-6

1. The Examiner further stated that, if Applicant could overcome the above 101 and 112, first paragraph rejection, claims 1 and 4-6 are still rejected under 35 U.S.C. § 112, first paragraph, because the specification, while being enabling for a polynucleotide of SEQ ID NO:2, does not reasonably provide enablement for a polynucleotide "encoding" SEQ ID NO:1. The specification discloses isolation of SEQ ID NO:2 which is over-expressed in a prostate cancer cell line. However, the Examiner stated, there is no evidence that the deduced SEQ ID NO:1 is expressed in any tissue. Those of skill in the art recognize that expression of mRNA, specific for a tissue type, does not dictate nor predict the translation of such mRNA into a polypeptide. The Examiner then cited various art allegedly supporting her contention that the predictability of protein translation or the extent of translation is not solely contingent on mRNA expression due to the multitude of homeostatic factors affecting transcription and translation. See, in particular, Shantz and Pegg (1999); McClean and Hill (1993); Fu et al. (1996); and Yokota et al. (1988), Office Action, p. 17.

Applicants Response

Applicants first of all point that the polynucleotide encoding SEQ ID NO:1 is enabled by way of the well established utility of STEAPRP (SEQ ID NO:1) as a STEAP related protein for the reasons previously addressed in the rejection of claims under 35 U.S.C. § 101.

With respect to the issue of whether the polypeptide encoded by the claimed polynucleotide is expressed in nature and/or at a level sufficient for its intended use, the Office Action has set forth the novel theory that the central dogma of molecular biology (*i.e.*, DNA directs transcription of messenger

RNA which in turn directs translation of protein) somehow does not apply to the discoveries of the present application. That is, the nucleotide sequence of SEQ ID NO:2 (which encodes the polypeptide of SEQ ID NO:1) was determined from a human cDNA library. That cDNA library in turn was made from messenger RNA isolated from human tissue. See the Specification, for example, at pages 25 to 26. Thus, the nucleotide sequences of the present invention are expressed sequences. The Office Action purports that the existence of an expressed mRNA does not insure that the protein encoded by the mRNA will be translated and, hence, the claimed subject matter lacks patentable utility.

Regulation of gene expression occurs at many levels, including transcription, splicing, polyadenylation, mRNA stability, mRNA transport and compartmentalization, translation efficiency, protein modification and protein turnover. While steady state mRNA levels are not always directly proportional to the amount of protein produced in a cell, mRNA levels are **routinely** used as an indicator of protein expression. Countless scientific publication have been based on data relating to mRNA levels when the polypeptide encoded by the mRNA was unknown or difficult to detect. Moreover, mRNA levels are **usually** a good indicator of protein levels in a cell. The Office Action cites several examples of protein regulation downstream of transcription; however, these examples represent comparatively unusual mechanisms of gene regulation. According to B. Lewin [(1997) Genes VI Oxford University Press, Inc. New York, NY] (pages attached as Exhibit F):

Transcription of a gene in the active state is controlled at the stage of initiation, that is, by the interaction of RNA polymerase with its promoter. This is now becoming susceptible to study in the *in vitro* systems... ***For most genes, this is a major control point; probably it is the most common level of regulation.*** [page 847, emphasis added].  
But having acknowledged that control of gene expression can occur at multiple stages, and that production of RNA cannot inevitably be equated with production of protein, it is clear that ***the overwhelming majority of regulatory events occur at the initiation of transcription. Regulation of tissue-specific gene transcription lies at the heart of eukaryotic differentiation.*** [pages 847-848, emphasis added]

Thus the question is not whether there is the potential for post-transcriptional regulation of SEQ ID NO:1 expression but whether one skilled in the art would have a reasonable expectation that SEQ ID NO:1 expression correlates with the levels of SEQ ID NO:2 mRNA. Applicants need only prove a "substantial likelihood" of utility; certainty is not required. *Brenner v. Manson*, 383 U.S. 519, 532, 148 USPQ 689 (1966). In the case of the instant invention, one skilled in the art would be imprudent in assuming, *a priori*, that protein levels did not correspond to mRNA levels and that levels of SEQ ID NO:1 were controlled predominantly in a post-transcriptional manner, thereby dismissing the significance of mRNA levels.

Applicants therefore submit that for the above reasons, the polynucleotide encoding SEQ ID NO:1 is enabled both by the well established utility of STEAPRP as a STEAP-related protein, as well as by the differential expression of its encoding polynucleotide, SEQ ID NO:2, in prostate cancer and prostate hyperplasia, therefore providing a substantial likelihood that the protein of SEQ ID NO:1 is similarly differentially expressed.

2. The Examiner stated that if Applicant could overcome the above 101 and 112, first paragraph rejection, claims 1-6 are still rejected under 35 U.S.C. § 112, first paragraph, because the specification, while being enabling for SEQ ID NO:2, does not reasonably provide enablement for the "complement" of SEQ ID NO:2. It is noted, the Examiner stated, that a complement could be partial or complete complement, wherein the partial complement could share with SEQ ID NO:2 only a few nucleotides. The Examiner stated, therefore, that the claims encompass non-disclosed nucleic acid sequences attached to SEQ ID NO:2, that is polynucleotide that are complements of SEQ ID NO:2 or 3 or a nucleic acid sequence encoding SEQ ID NO:1, and that when given the broadest reasonable interpretation, the claims are clearly intended to encompass a variety of species including full-length cDNAs, genes and protein coding regions. It would be expected that a substantial number of hybridizing molecules encompassed by the claims would not share either structural or functional properties with SEQ ID NO:2.

Applicants Response

As previously discussed in this response, the definition of the "complement" of a cDNA at p. 6, lines 27-29 of the specification recites a cDNA "---which is completely complementary over its full length---", and therefore that the use of the term, as recited in the claims, explicitly excludes "partial complementarity. The claims therefore do not encompass sequences "attached to SEQ ID NO:2 or 3 or a nucleic acid encoding SEQ ID NO:1" that are anything less than the full complement of these sequences and which would therefore share only identical structural or functional properties of these nucleic acid sequences.

For all of the above reasons, applicants submit that claims 1-6 are fully enabled by the specification, and therefore request withdrawal of the rejection of these claims under 35 U.S.C. § 112, first paragraph.

35 U.S.C. § 102(b), Rejection of Claims 1-2

The Examiner has rejected claims 1-2 under 35 U.S.C § 102(a) as being anticipated by PN=6329503 or Gattung et al., GenBank sequence data base (Accession AC002064). The Examiner

stated that the claims are drawn to a "complement" of a cDNA encoding SEQ ID NO:1 or to a nucleic acid sequence of SEQ ID NO:2 or 3.

PN=6329503 teaches a polypeptide which is 100% similar to SEQ ID NO:1 from amino acid 246 to amino acid 418 of SEQ ID NO:1. Gattung et al. teach a polynucleotide which is 99.8% similar to SEQ ID NO:2 from nucleotide 804 to 1338, and a polynucleotide sequence which is 99.6% similar to SEQ ID NO:3 from nucleotide 280 to 508. Given these polynucleotides, the Examiner stated, one of ordinary skill in the art would immediately envision the claimed polynucleotide.

Applicants Response

Claim 1 has been amended to recite a cDNA, or the complement thereof, encoding SEQ ID NO:1, specified antigenic fragments of SEQ ID NO:1, a specified biologically active portion of SEQ ID NO:1, and a variant of SEQ ID NO:1 having at least 95% sequence identity to SEQ ID NO:1. Claim 2 has likewise been amended to recite a cDNA comprising SEQ ID NO:2, a variant to SEQ ID NO:2 having at least 85% sequence identity to SEQ ID NO:2, or a fragment of SEQ ID NO:1 consisting of SEQ ID NO:3. Neither PN=6329503 nor Gattung et al. anticipate any of the above recited polynucleotides or their complete complements, and Applicants therefore request withdrawal of the rejection of claims 1-2 under 35 U.S.C. § 102(a).

CONCLUSION

In light of the above amendments and remarks, Applicants submit that the present application is fully in condition for allowance, and request that the Examiner withdraw the outstanding rejections. Early notice to that effect is earnestly solicited. Applicants further request that, upon allowance of claims 1 and 3, claims 7-12 be rejoined and examined as methods of use of the polynucleotides of claims 1 and 3 that depend from and are of the same scope as claims 1 and 3 in accordance with *In re Ochiai and Brouwer* and the MPEP § 1801.04.

If the Examiner contemplates other action, or if a telephone conference would expedite allowance of the claims, Applicants invite the Examiner to contact Applicants' Attorney at (650) 855-0555.

Applicants believe that no fee is due with this communication. However, if the USPTO determines that a fee is due, the Commissioner is hereby authorized to charge Deposit Account No. 09-0108.

Respectfully submitted,

INCYTE CORPORATION

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March 28, 2003

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likely, its power can be attributed to its incorporation of more information than any other measure; it takes account of the full substitution and gap data (like raw scores) but also has details about the sequence lengths and composition and is scaled appropriately.

We find that statistical scores are not only powerful, but also easy to interpret. SSEARCH and FASTA show close agreement between statistical scores and actual number of errors per query (Fig. 4). The expectation value score gives a good, slightly conservative estimate of the chances of the two sequences being found at random in a given query. Thus, an E-value of 0.01 indicates that roughly one pair of nonhomologs of this similarity should be found in every 100 different queries. Neither raw scores nor percentage identity can be interpreted in this way, and these results validate the suitability of the extreme value distribution for describing the scores from a database search.

The P-values from BLAST also should be directly interpretable but were found to overstate significance by more than two orders of magnitude for 1% EPQ for this database. Nonetheless, these results strongly suggest that the analytic theory is fundamentally appropriate. WU-BLAST2 scores were more reliable than those from BLAST, but also exaggerate expected confidence by more than an order of magnitude at 1% EPQ.

**Overall Detection of Homologs and Comparison of Algorithms.** The results in Fig. 5A and Table 1 show that pairwise sequence comparison is capable of identifying only a small fraction of the homologous pairs of sequences in PDB40D-B. Even SSEARCH with E-values, the best protocol tested, could find only 18% of all relationships at a 1% EPQ. BLAST, which identifies 15%, was the worst performer, whereas FASTA  $k_{\text{up}} = 1$  is nearly as effective as SSEARCH. FASTA  $k_{\text{up}} = 2$  and WU-BLAST2 are intermediate in their ability to detect homologs. Comparison of different algorithms indicates that those capable of identifying more homologs are generally slower. SSEARCH is 25 times slower than BLAST and 6.5 times slower than FASTA  $k_{\text{up}} = 1$ . WU-BLAST2 is slightly faster than FASTA  $k_{\text{up}} = 2$ , but the latter has more interpretable scores.

In PDB90D-B, where there are many close relationships, the best method can identify only 38% of structurally known homologs (Fig. 5B). The method which finds that many relationships is WU-BLAST2. Consequently, we infer that the differences between FASTA  $k_{\text{up}} = 1$ , SSEARCH, and WU-BLAST2 programs are unlikely to be significant when compared with variation in database composition and scoring reliability.

Fig. 6 helps to explain why most distant homologs cannot be found by sequence comparison: a great many such relationships have no more sequence identity than would be expected by chance. SSEARCH with E-values can recognize >90% of the homologous pairs with 30–40% identity. In this region, there are 30 pairs of homologous proteins that do not have significant E-values, but 26 of these involve sequences with <50 residues. Of sequences having 25–30% identity, 75% are identified by SSEARCH E-values. However, although the number of homologs grows at lower levels of identity, the detection falls off sharply: only 40% of homologs with 20–25% identity

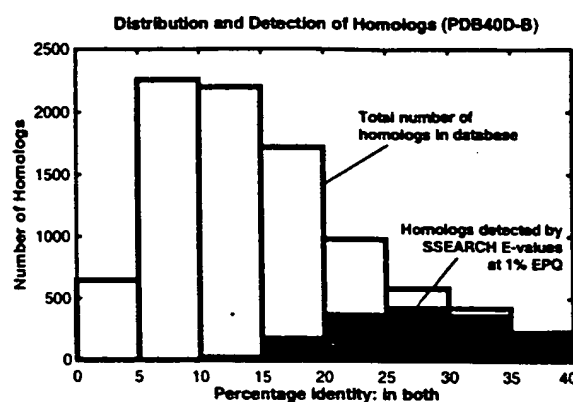


FIG. 6. Distribution and detection of homologs in PDB40D-B. Bars show the distribution of homologous pairs PDB40D-B according to their identity (using the measure of identity in both). Filled regions indicate the number of these pairs found by the best database searching method (SSEARCH with E-values) at 1% EPQ. The PDB40D-B database contains proteins with <40% identity, and as shown on this graph, most structurally identified homologs in the database have diverged extremely far in sequence and have <20% identity. Note that the alignments may be inaccurate, especially at low levels of identity. Filled regions show that SSEARCH can identify most relationships that have 25% or more identity, but its detection wanes sharply below 25%. Consequently, the great sequence divergence of most structurally identified evolutionary relationships effectively defeats the ability of pairwise sequence comparison to detect them.

are detected and only 10% of those with 15–20% can be found. These results show that statistical scores can find related proteins whose identity is remarkably low; however, the power of the method is restricted by the great divergence of many protein sequences.

After completion of this work, a new version of pairwise BLAST was released: BLASTGP (37). It supports gapped alignments, like WU-BLAST2, and dispenses with sum statistics. Our initial tests on BLASTGP using default parameters show that its E-values are reliable and that its overall detection of homologs was substantially better than that of ungapped BLAST, but not quite equal to that of WU-BLAST2.

## CONCLUSION

The general consensus amongst experts (see refs. 7, 24, 25, 27 and references therein) suggests that the most effective sequence searches are made by (i) using a large current database in which the protein sequences have been complexity masked and (ii) using statistical scores to interpret the results. Our experiments fully support this view.

Our results also suggest two further points. First, the E-values reported by FASTA and SSEARCH give fairly accurate estimates of the significance of each match, but the P-values provided by BLAST and WU-BLAST2 underestimate the true

Table 1. Summary of sequence comparison methods with PDB40D-B

Method	Relative Time*	1% EPQ Cutoff	Coverage at 1% EPQ
SSEARCH % identity: within alignment	25.5	>70%	<0.1
SSEARCH % identity: within both	25.5	34%	3.0
SSEARCH % identity: HSSP-scaled	25.5	35% (HSSP + 9.8)	4.0
SSEARCH Smith-Waterman raw scores	25.5	142	10.5
SSEARCH E-values	25.5	0.03	18.4
FASTA $k_{\text{up}} = 1$ E-values	3.9	0.03	17.9
FASTA $k_{\text{up}} = 2$ E-values	1.4	0.03	16.7
WU-BLAST2 P-values	1.1	0.003	17.5
BLAST P-values	1.0	0.00016	14.8

\*Times are from large database searches with genome proteins.



extent of errors. Second, SSEARCH, WU-BLAST2, and FASTA ktup = 1 perform best, though BLAST and FASTA ktup = 2 detect most of the relationships found by the best procedures and are appropriate for rapid initial searches.

The homologous proteins that are found by sequence comparison can be distinguished with high reliability from the huge number of unrelated pairs. However, even the best database searching procedures tested fail to find the large majority of distant evolutionary relationships at an acceptable error rate. Thus, if the procedures assessed here fail to find a reliable match, it does not imply that the sequence is unique; rather, it indicates that any relatives it might have are distant ones.\*\*

\*\*Additional and updated information about this work, including supplementary figures, may be found at <http://sss.stanford.edu/sss/>.

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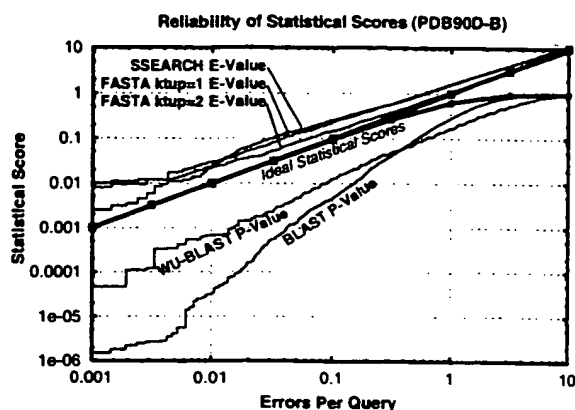


FIG. 4. Reliability of statistical scores in PDB90D-B: Each line shows the relationship between reported statistical score and actual error rate for a different program. E-values are reported for SSEARCH and FASTA, whereas P-values are shown for BLAST and WU-BLAST2. If the scoring were perfect, then the number of errors per query and the E-values would be the same, as indicated by the upper bold line. (P-values should be the same as EPQ for small numbers, and diverges at higher values, as indicated by the lower bold line.) E-values from SSEARCH and FASTA are shown to have good agreement with EPQ but underestimate the significance slightly. BLAST and WU-BLAST2 are overconfident, with the degree of exaggeration dependent upon the score. The results for PDB40D-B were similar to those for PDB90D-B despite the difference in number of homologs detected. This graph could be used to roughly calibrate the reliability of a given statistical score.

ignored in previous tests but is essential for the straightforward or automatic interpretation of sequence comparison results. Further, it provides a clear indication of the confidence that should be ascribed to each match. Indeed, the EPQ measure should approximate the expectation value reported by database searching programs, if the programs' estimates are accurate.

**The Performance of Scoring Schemes.** All of the programs tested could provide three fundamental types of scores. The first score is the percentage identity, which may be computed in several ways based on either the length of the alignment or the lengths of the sequences. The second is a "raw" or "Smith-Waterman" score, which is the measure optimized by the Smith-Waterman algorithm and is computed by summing the substitution matrix scores for each position in the alignment and subtracting gap penalties. In BLAST, a measure

related to this score is scaled into bits. Third is a statistical score based on the extreme value distribution. These results are summarized in Fig. 1.

**Sequence Identity.** Though it has been long established that percentage identity is a poor measure (35), there is a common rule-of-thumb stating that 30% identity signifies homology. Moreover, publications have indicated that 25% identity can be used as a threshold (17, 36). We find that these thresholds, originally derived years ago, are not supported by present results. As databases have grown, so have the possibilities for chance alignments with high identity; thus, the reported cutoffs lead to frequent errors. Fig. 2 shows one of the many pairs of proteins with very different structures that nonetheless have high levels of identity over considerable aligned regions. Despite the high identity, the raw and the statistical scores for such incorrect matches are typically not significant. The principal reasons percentage identity does so poorly seem to be that it ignores information about gaps and about the conservative or radical nature of residue substitutions.

From the PDB90D-B analysis in Fig. 3, we learn that 30% identity is a reliable threshold for this database only for sequence alignments of at least 150 residues. Because one unrelated pair of proteins has 43.5% identity over 62 residues, it is probably necessary for alignments to be at least 70 residues in length before 40% is a reasonable threshold, for a database of this particular size and composition.

At a given reliability, scores based on percentage identity detect just a fraction of the distant homologs found by statistical scoring. If one measures the percentage identity in the aligned regions without consideration of alignment length, then a negligible number of distant homologs are detected. Use of the HSP equation improves the value of percentage identity, but even this measure can find only 4% of all known homologs at 1% EPQ. In short, percentage identity discards most of the information measured in a sequence comparison.

**Raw Scores.** Smith-Waterman raw scores perform better than percentage identity (Fig. 1), but ln-scaling (7) provided no notable benefit in our analysis. It is necessary to be very precise when using either raw or bit scores because a 20% change in cutoff score could yield a tenfold difference in EPQ. However, it is difficult to choose appropriate thresholds because the reliability of a bit score depends on the lengths of the proteins matched and the size of the database. Raw score thresholds also are affected by matrix and gap parameters.

**Statistical Scores.** Statistical scores were introduced partly to overcome the problems that arise from raw scores. This scoring scheme provides the best discrimination between homologous proteins and those which are unrelated. Most

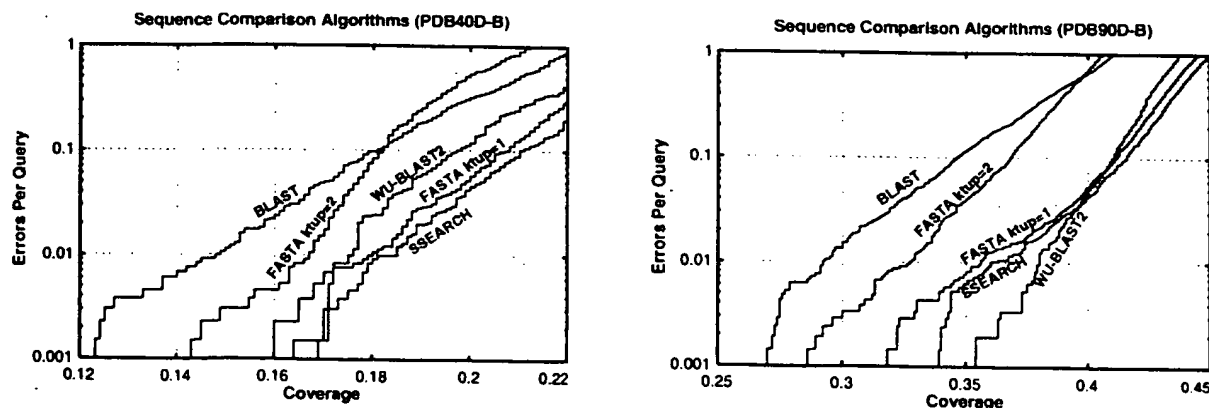


FIG. 5. Coverage vs. error plots of different sequence comparison methods: Five different sequence comparison methods are evaluated, each using statistical scores (E- or P-values). (A) PDB40D-B database. In this analysis, the best method is the slow SSEARCH, which finds 18% of relationships at 1% EPQ. FASTA ktup = 1 and WU-BLAST2 are almost as good. (B) PDB90D-B database. The quick WU-BLAST2 program provides the best coverage at 1% EPQ on this database, although at higher levels of error it becomes slightly worse than FASTA ktup = 1 and SSEARCH.

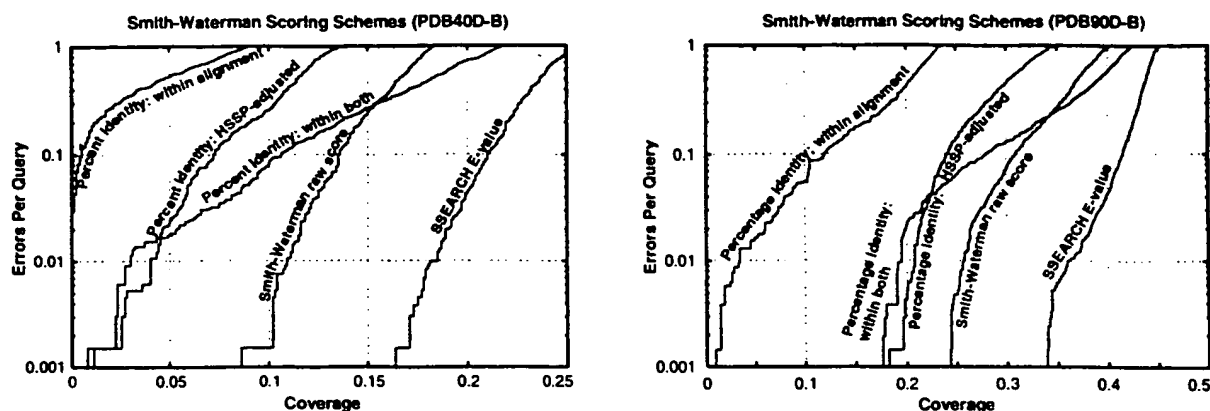


FIG. 1. Coverage vs. error plots of different scoring schemes for sSEARCH Smith-Waterman. (A) Analysis of PDB40D-B database. (B) Analysis of PDB90D-B database. All of the proteins in the database were compared with each other using the sSEARCH program. The results of this single set of comparisons were considered using five different scoring schemes and assessed. The graphs show the coverage and errors per query (EPQ) for statistical scores, raw scores, and three measures using percentage identity. In the coverage vs. error plot, the x axis indicates the fraction of all homologs in the database (known from structure) which have been detected. Precisely, it is the number of detected pairs of proteins with the same fold divided by the total number of pairs from a common superfamily. PDB40D-B contains a total of 9,044 homologs, so a score of 10% indicates identification of 904 relationships. The y axis reports the number of EPQ. Because there are 1,323 queries made in the PDB40D-B all-vs.-all comparison, 13 errors corresponds to 0.01, or 1% EPQ. The y axis is presented on a log scale to show results over the widely varying degrees of accuracy which may be desired. The scores that correspond to the levels of EPQ and coverage are shown in Fig. 4 and Table 1. The graph demonstrates the trade-off between sensitivity and selectivity. As more homologs are found (moving to the right), more errors are made (moving up). The ideal method would be in the lower right corner of the graph, which corresponds to identifying many evolutionary relationships without selecting unrelated proteins. Three measures of percentage identity are plotted. Percentage identity within alignment is the degree of identity within the aligned region of the proteins, without consideration of the alignment length. Percentage identity within both is the number of identical residues in the aligned region as a percentage of the average length of the query and target proteins. The HSSP equation (17) is  $H = 290.15l^{-0.562}$  where  $l$  is length for  $10 < l < 80$ ;  $H > 100$  for  $l < 10$ ;  $H = 24.7$  for  $l > 80$ . The percentage identity HSSP-adjusted score is the percent identity within the alignment minus  $H$ . Smith-Waterman raw scores and E-values were taken directly from the sequence comparison program.

perfect separation, with all of the homologs at the top of the list and unrelated proteins below. In practice, perfect separation is impossible to achieve so instead one is interested in drawing a threshold above which there are the largest number of related pairs of sequences consistent with an acceptable error rate.

Our procedure involved measuring the coverage and error for every threshold. Coverage was defined as the fraction of structurally determined homologs that have scores above the selected threshold; this reflects the sensitivity of a method. Errors per query (EPQ), an indicator of selectivity, is the number of nonhomologous pairs above the threshold divided by the number of queries. Graphs of these data, called coverage vs. error plots, were devised to understand how

protocols compare at different levels of accuracy. These graphs share effectively all of the beneficial features of Receiver Operating Characteristic (ROC) plots (33, 34) but better represent the high degrees of accuracy required in sequence comparison and the huge background of nonhomologs.

This assessment procedure is directly relevant to practical sequence database searching, for it provides precisely the information necessary to perform a reliable sequence database search. The EPQ measure places a premium on score consistency; that is, it requires scores to be comparable for different queries. Consistency is an aspect which has been largely

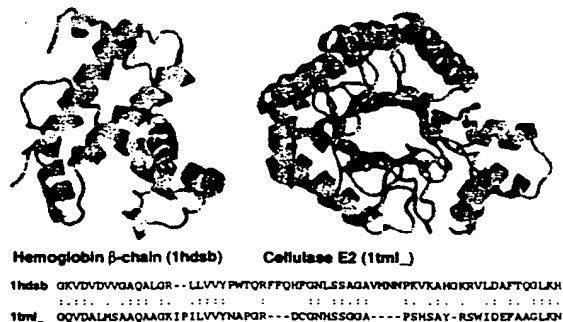


FIG. 2. Unrelated proteins with high percentage identity. Hemoglobin  $\beta$ -chain (PDB code 1hdsb chain b, ref. 38, Left) and cellulase E2 (PDB code 1tml, ref. 39, Right) have 39% identity over 64 residues, a level which is often believed to be indicative of homology. Despite this high degree of identity, their structures strongly suggest that these proteins are not related. Appropriately, neither the raw alignment score of 85 nor the E-value of 1.3 is significant. Proteins rendered by RASMOL (40).

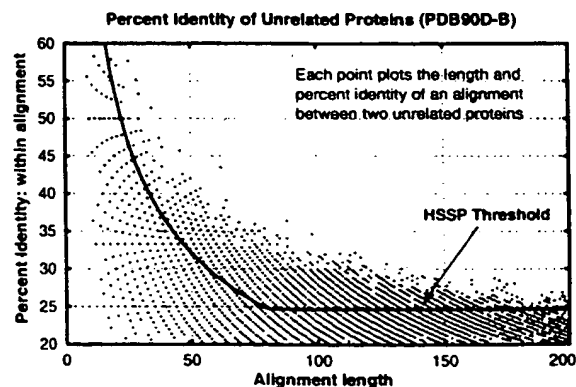


FIG. 3. Length and percentage identity of alignments of unrelated proteins in PDB90D-B: Each pair of nonhomologous proteins found with sSEARCH is plotted as a point whose position indicates the length and the percentage identity within the alignment. Because alignment length and percentage identity are quantized, many pairs of proteins may have exactly the same alignment length and percentage identity. The line shows the HSSP threshold (though it is intended to be applied with a different matrix and parameters).

## Assessing sequence comparison methods with reliable structurally identified distant evolutionary relationships

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**ABSTRACT** Pairwise sequence comparison methods have been assessed using proteins whose relationships are known reliably from their structures and functions, as described in the SCOP database [Murzin, A. G., Brenner, S. E., Hubbard, T. & Chothia C. (1995) *J. Mol. Biol.* 247, 536–540]. The evaluation tested the programs BLAST [Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) *J. Mol. Biol.* 215, 403–410], WU-BLAST2 [Altschul, S. F. & Gish, W. (1996) *Methods Enzymol.* 266, 460–480], FASTA [Pearson, W. R. & Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. USA* 85, 2444–2448], and SSEARCH [Smith, T. F. & Waterman, M. S. (1981) *J. Mol. Biol.* 147, 195–197] and their scoring schemes. The error rate of all algorithms is greatly reduced by using statistical scores to evaluate matches rather than percentage identity or raw scores. The E-value statistical scores of SSEARCH and FASTA are reliable: the number of false positives found in our tests agrees well with the scores reported. However, the P-values reported by BLAST and WU-BLAST2 exaggerate significance by orders of magnitude. SSEARCH, FASTA  $ktup = 1$ , and WU-BLAST2 perform best, and they are capable of detecting almost all relationships between proteins whose sequence identities are >30%. For more distantly related proteins, they do much less well; only one-half of the relationships between proteins with 20–30% identity are found. Because many homologs have low sequence similarity, most distant relationships cannot be detected by any pairwise comparison method; however, those which are identified may be used with confidence.

Sequence database searching plays a role in virtually every branch of molecular biology and is crucial for interpreting the sequences issuing forth from genome projects. Given the method's central role, it is surprising that overall and relative capabilities of different procedures are largely unknown. It is difficult to verify algorithms on sample data because this requires large data sets of proteins whose evolutionary relationships are known unambiguously and independently of the methods being evaluated. However, nearly all known homologs have been identified by sequence analysis (the method to be tested). Also, it is generally very difficult to know, in the absence of structural data, whether two proteins that lack clear sequence similarity are unrelated. This has meant that although previous evaluations have helped improve sequence comparison, they have suffered from insufficient, imperfectly characterized, or artificial test data. Assessment also has been problematic because high quality database sequence searching attempts to have both sensitivity (detection of homologs) and specificity (rejection of unrelated proteins); however, these complementary goals are linked such that increasing one causes the other to be reduced.

Sequence comparison methodologies have evolved rapidly, so no previously published tests have evaluated modern versions of programs commonly used. For example, parameters in BLAST (1) have changed, and WU-BLAST2 (2)—which produces gapped alignments—has become available. The latest version of FASTA (3) previously tested was 1.6, but the current release (version 3.0) provides fundamentally different results in the form of statistical scoring.

The previous reports also have left gaps in our knowledge. For example, there has been no published assessment of thresholds for scoring schemes more sophisticated than percentage identity. Thus, the widely discussed statistical scoring measures have never actually been evaluated on large databases of real proteins. Moreover, the different scoring schemes commonly in use have not been compared.

Beyond these issues, there is a more fundamental question: in an absolute sense, how well does pairwise sequence comparison work? That is, what fraction of homologous proteins can be detected using modern database searching methods?

In this work, we attempt to answer these questions and to overcome both of the fundamental difficulties that have hindered assessment of sequence comparison methodologies. First, we use the set of distant evolutionary relationships in the SCOP: Structural Classification of Proteins database (4), which is derived from structural and functional characteristics (5). The SCOP database provides a uniquely reliable set of homologs, which are known independently of sequence comparison. Second, we use an assessment method that jointly measures both sensitivity and specificity. This method allows straightforward comparison of different sequence searching procedures. Further, it can be used to aid interpretation of real database searches and thus provide optimal and reliable results.

**Previous Assessments of Sequence Comparison.** Several previous studies have examined the relative performance of different sequence comparison methods. The most encompassing analyses have been by Pearson (6, 7), who compared the three most commonly used programs. Of these, the Smith–Waterman algorithm (8) implemented in SSEARCH (3) is the oldest and slowest but the most rigorous. Modern heuristics have provided BLAST (1) the speed and convenience to make it the most popular program. Intermediate between these two is FASTA (3), which may be run in two modes offering either greater speed ( $ktup = 2$ ) or greater effectiveness ( $ktup = 1$ ). Pearson also considered different parameters for each of these programs.

To test the methods, Pearson selected two representative proteins from each of 67 protein superfamilies defined by the PIR database (9). Each was used as a query to search the database, and the matched proteins were marked as being homologous or unrelated according to their membership of PIR

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Abbreviation: EPQ, errors per query.

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superfamilies. Pearson found that modern matrices and "ln-scaling" of raw scores improve results considerably. He also reported that the rigorous Smith-Waterman algorithm worked slightly better than FASTA, which was in turn more effective than BLAST.

Very large scale analyses of matrices have been performed (10), and Henikoff and Henikoff (11) also evaluated the effectiveness of BLAST and FASTA. Their test with BLAST considered the ability to detect homologs above a predetermined score but had no penalty for methods which also reported large numbers of spurious matches. The Henikoffs searched the SWISS-PROT database (12) and used PROSITE (13) to define homologous families. Their results showed that the BLOSUM62 matrix (14) performed markedly better than the extrapolated PAM-series matrices (15), which previously had been popular.

A crucial aspect of any assessment is the data that are used to test the ability of the program to find homologs. But in Pearson's and the Henikoffs' evaluations of sequence comparison, the correct results were effectively unknown. This is because the superfamilies in PIR and PROSITE are principally created by using the same sequence comparison methods which are being evaluated. Interdependency of data and methods creates a "chicken and egg" problem, and means for example, that new methods would be penalized for correctly identifying homologs missed by older programs. For instance, immunoglobulin variable and constant domains are clearly homologous, but PIR places them in different superfamilies. The problem is widespread: each superfamily in PIR 48.00 with a structural homolog is itself homologous to an average of 1.6 other PIR superfamilies (16).

To surmount these sorts of difficulties, Sander and Schneider (17) used protein structures to evaluate sequence comparison. Rather than comparing different sequence comparison algorithms, their work focused on determining a length-dependent threshold of percentage identity, above which all proteins would be of similar structure. A result of this analysis was the HSP equation; it states that proteins with 25% identity over 80 residues will have similar structures, whereas shorter alignments require higher identity. (Other studies also have used structures (18–20), but these focused on a small number of model proteins and were principally oriented toward evaluating alignment accuracy rather than homology detection.)

A general solution to the problem of scoring comes from statistical measures (i.e., E-values and P-values) based on the extreme value distribution (21). Extreme value scoring was implemented analytically in the BLAST program using the Karlin and Altschul statistics (22, 23) and empirical approaches have been recently added to FASTA and SSEARCH. In addition to being heralded as a reliable means of recognizing significantly similar proteins (24, 25), the mathematical tractability of statistical scores "is a crucial feature of the BLAST algorithm" (1). The validity of this scoring procedure has been tested analytically and empirically (see ref. 2 and references in ref. 24). However, all large empirical tests used random sequences that may lack the subtle structure found within biological sequences (26, 27) and obviously do not contain any real homologs. Thus, although many researchers have suggested that statistical scores be used to rank matches (24, 25, 28), there have been no large rigorous experiments on biological data to determine the degree to which such rankings are superior.

**A Database for Testing Homology Detection.** Since the discovery that the structures of hemoglobin and myoglobin are very similar though their sequences are not (29), it has been apparent that comparing structures is a more powerful (if less convenient) way to recognize distant evolutionary relationships than comparing sequences. If two proteins show a high degree of similarity in their structural details and function, it

is very probable that they have an evolutionary relationship though their sequence similarity may be low.

The recent growth of protein structure information combined with the comprehensive evolutionary classification in the SCOP database (4, 5) have allowed us to overcome previous limitations. With these data, we can evaluate the performance of sequence comparison methods on real protein sequences whose relationships are known confidently. The SCOP database uses structural information to recognize distant homologs, the large majority of which can be determined unambiguously. These superfamilies, such as the globins or the immunoglobulins, would be recognized as related by the vast majority of the biological community despite the lack of high sequence similarity.

From SCOP, we extracted the sequences of domains of proteins in the Protein Data Bank (PDB) (30) and created two databases. One (PDB90D-B) has domains, which were all <90% identical to any other, whereas (PDB40D-B) had those <40% identical. The databases were created by first sorting all protein domains in SCOP by their quality and making a list. The highest quality domain was selected for inclusion in the database and removed from the list. Also removed from the list (and discarded) were all other domains above the threshold level of identity to the selected domain. This process was repeated until the list was empty. The PDB40D-B database contains 1,323 domains, which have 9,044 ordered pairs of distant relationships, or ~0.5% of the total 1,749,006 ordered pairs. In PDB90D-B, the 2,079 domains have 53,988 relationships, representing 1.2% of all pairs. Low complexity regions of sequence can achieve spurious high scores, so these were masked in both databases by processing with the SEG program (27) using recommended parameters: 12 1.8 2.0. The databases used in this paper are available from <http://sss.stanford.edu/sss/>, and databases derived from the current version of SCOP may be found at <http://scop.mrc-lmb.cam.ac.uk/scop/>.

Analyses from both databases were generally consistent, but PDB40D-B focuses on distantly related proteins and reduces the heavy overrepresentation in the PDB of a small number of families (31, 32), whereas PDB90D-B (with more sequences) improves evaluations of statistics. Except where noted otherwise, the distant homolog results here are from PDB40D-B. Although the precise numbers reported here are specific to the structural domain databases used, we expect the trends to be general.

**Assessment Data and Procedure.** Our assessment of sequence comparison may be divided into four different major categories of tests. First, using just a single sequence comparison algorithm at a time, we evaluated the effectiveness of different scoring schemes. Second, we assessed the reliability of scoring procedures, including an evaluation of the validity of statistical scoring. Third, we compared sequence comparison algorithms (using the optimal scoring scheme) to determine their relative performance. Fourth, we examined the distribution of homologs and considered the power of pairwise sequence comparison to recognize them. All of the analyses used the databases of structurally identified homologs and a new assessment criterion.

The analyses tested BLAST (1), version 1.4.9MP, and WU-BLAST2 (2), version 2.0a13MP. Also assessed was the FASTA package, version 3.0i76 (3), which provided FASTA and the SSEARCH implementation of Smith-Waterman (8). For SSEARCH and FASTA, we used BLOSUM45 with gap penalties -12/-1 (7, 16). The default parameters and matrix (BLOSUM62) were used for BLAST and WU-BLAST2.

**The "Coverage Vs. Error" Plot.** To test a particular protocol (comprising a program and scoring scheme), each sequence from the database was used as a query to search the database. This yielded ordered pairs of query and target sequences with associated scores, which were sorted, on the basis of their scores, from best to worst. The ideal method would have



# Regulation of Bcl-2 during androgen-unresponsive progression of prostate cancer

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The progression of prostate cancer from androgen-responsive to an androgen-unresponsive state remains the greatest obstacle in the treatment of this disease. Androgen-unresponsive prostate cancer is highly resistant to chemotherapy and radiation treatment that kill cells by the induction of apoptosis. Elucidating the molecular mechanisms of apoptosis regulation in prostate cancer can be useful in the development of new strategies for effective therapy of androgen-unresponsive cancer. We analyzed the Bcl-2 family of apoptosis regulators using various passages of the LNCaP prostate cancer cell line, which serve as an *in vitro* model for the progression of prostate cancer from androgen-responsive to androgen-unresponsive. In our model, progressively higher passages of LNCaP cells represent the progression to androgen-unresponsiveness. We examined the basal mRNA expression of the Bcl-2 family of apoptosis regulators. Under normal growth conditions, both androgen-responsive and androgen-unresponsive LNCaP cells express the Bcl-2 family of genes at similar levels. Western blot analysis showed the presence of Bcl-2 protein in androgen-responsive cells but not in androgen-unresponsive cells. Both androgen-responsive and androgen-unresponsive cells expressed Bax protein at similar levels. When exposed to oxidative stress, androgen-responsive cells underwent apoptosis but androgen-unresponsive cells exhibited resistance suggesting that the progression to androgen-unresponsiveness was associated with altered regulation of apoptosis. Treatment with paclitaxel or sodium butyrate induced apoptosis in both androgen-responsive and androgen-unresponsive cells suggesting that the apoptotic machinery is still intact in androgen-unresponsive LNCaP cells.

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**Keywords:** apoptosis; prostate cancer; LNCaP; Bcl-2; androgen-unresponsive

## Introduction

Prostate cancer is the second leading cause of cancer death in men and one of the most therapy resistant cancers.<sup>1</sup> Few therapies are available for the treatment of advanced states of prostate cancer because the cancer cells are androgen-independent and the primary therapy

of androgen ablation ceases to have an effect.<sup>2</sup> Chemotherapy is a common strategy used to treat various forms of cancer, including prostate cancer. The mechanism by which many chemotherapeutic drugs act is by targeting cancer cells to undergo apoptosis. Most prostate cancers initially respond to androgen ablation by undergoing apoptosis but this therapy selects for cancer cells that are androgen insensitive.<sup>3</sup> Interestingly, these androgen insensitive cells still maintain the proper apoptotic machinery suggesting that these cells may be targets for novel chemotherapeutic agents.

Recent studies indicate that in prostate cancer aberrant expression of important apoptosis proteins, such as Bcl-2 and Bax, may be responsible for the androgen refractory phenotype.<sup>4</sup> Bcl-2 is a potent anti-apoptotic protein that regulates the release of cytochrome C from the mitochondria and Bax is an important pro-apoptotic protein that is

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known to heterodimerize with Bcl-2 and affect the ability of Bcl-2 to block apoptosis.<sup>5</sup> Studies have shown that normal prostate cells express little or no Bcl-2 whereas prostate cancer cells and metastatic prostate cancer cell lines such as DU145, PC3, and LNCaP express various amounts of this protein.<sup>6-8</sup>

The LNCaP prostate cancer cell line is one of the few androgen-responsive cell lines available for the study of androgen-responsive prostate cancer. Often, LNCaP cells are compared to androgen-unresponsive cell lines such as DU145 or PC3 to draw comparisons between androgen-responsive and androgen-unresponsive characteristics. The limitations of such comparisons include the fact that each of these three cell lines were derived from different sources and comparisons between the three cannot take into account the diversity of different genetic backgrounds. Understanding the molecular mechanisms during the progression of prostate cancer to an androgen-unresponsive state requires a cell model with the same genetic background. The androgen-responsive LNCaP prostate cancer cell line becomes androgen-unresponsive at high passage numbers,<sup>9</sup> providing a unique model to study the clinical progression of prostate cancer to an androgen-unresponsive state.

Among the prostate cancer cell lines, LNCaP cells exhibit a wild type p53, while DU145 cells contain two mutations in the p53 gene and the PC3 cells are null for p53.<sup>10</sup> Androgen receptor, prostate specific antigen and prostatic acid phosphatase are all expressed in LNCaP cells, though Lin *et al.*<sup>9</sup> showed that high passage androgen-independent LNCaP cells had decreased expression of prostatic acid phosphatase. The androgen receptor expressed in LNCaP cells contains a mutation in the hormone-binding domain that affects the response of these cells to steroid binding and to anti-androgens.<sup>11</sup> The result of this mutation in the androgen receptor in LNCaP cells causes the cells to respond with high affinity to estrogens and some anti-androgens.<sup>12</sup>

The LNCaP prostate cancer cell line maintains the proper apoptotic machinery and can be induced to undergo apoptosis. Tang *et al.*<sup>7</sup> showed that LNCaP cells express Bcl-2, Bax, Bcl-xL and Bak and are more sensitive to the induction of apoptosis when compared with DU145 and PC3 cells. Induction of apoptosis in LNCaP cells and an androgen-independent subline, LNCaP-AI, was shown to occur via activation of Rb.<sup>13</sup>

We investigated the regulation of expression of the Bcl-2 family of proteins during the progression towards androgen-unresponsiveness utilizing the LNCaP prostate cancer cell model. It had previously been reported that early passage LNCaP cells (passages 20-33) are androgen-dependent, while the later passages (passage 80 and higher) are androgen-independent.<sup>9</sup> We used an extension of this established model. In our model, LNCaP-R cells are from passages 20 to 50, are androgen-responsive, and slow in growth. LNCaP-RF cells are from passages 50 to 95, are somewhat androgen-responsive, and faster growing than LNCaP-R. LNCaP-UR cells comprise passages higher than 95, are androgen-unresponsive, and fast growing. Utilizing this model, we seek to determine what changes occur in the Bcl-2 family of apoptosis regulators that may contribute to the progression of prostate to an androgen-independent state.

## Materials and methods

### Cell culture

LNCaP, DU145, and PC3 cells were maintained in RPMI1640 supplemented with 7% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) (Gibco-BRL, Life Technologies, Inc. Grand Island, NY, USA). MCF7 cells were maintained in DMEM supplemented with 7% FBS and 1% PS (Life Technologies, Inc. Grand Island, NY, USA). MDAPCa 2A and 2B cells were maintained in BRFF media (Biological Research Faculty and Facility, Inc. Ijamsville, MD, USA) supplemented with 20% FBS and 1% PS. Cells were routinely fed with fresh media, passaged as needed, and maintained in a 37°C incubator at 5% CO<sub>2</sub>.

### DHT stimulation experiment

LNCaP cells were treated with dihydrotestosterone (DHT) (Sigma Chemical Co, St Louis, MO, USA) and growth stimulation was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co.) assay. Prior to measuring DHT stimulation, a standard curve of various cell numbers and absorbance from the MTT assay was generated. A total of 20 000, 40 000, 60 000, 80 000, 100 000 and 120 000 cells were seeded into six-well plates from each of the representative LNCaP cells. Cells were allowed to sit down for 48 h then processed for MTT assay. For MTT assay, cells were rinsed once with phenol red-free HBSS and 2.0 ml MTT (dissolved in serum-free, phenol red-free RPMI 1640) was added to each well and placed in a 37°C incubator for 4 h. After the incubation period, MTT reagent was removed and 2.0 ml of 99% isopropanol was added to dissolve the product. A total of 100 µl of each sample was placed into a 96-well plate and the absorbance was read at 590 nm using a Spectra Max 190 (Molecular Devices, Sunnyvale, CA, USA).

For DHT stimulation, a total of  $3 \times 10^4$  cells from each representative of the LNCaP model (R, RF and UR) were seeded into 6-well plates using RPMI 1640 supplemented with 7% FBS and 1% PS. After 2 days the media was aspirated, the cells were rinsed once with phenol red-free HBSS, and phenol red-free RPMI 1640 containing 2% charcoal stripped FBS was added. After 1 day, the media was aspirated and cells (in triplicate) were fed with phenol red-free RPMI 1640 with 2% charcoal stripped FBS and  $1 \times 10^{-8}$  M DHT. Control cells (in triplicate) were fed with phenol red-free RPMI 1640 with 2% charcoal stripped FBS. MTT assay was performed after 4 days of incubation with DHT.

### RNase protection assay

RNase protection assays were performed using Ribo-Quant Ribonuclease protection assay (RPA) kit, (Pharmingen, San Diego, CA, USA). Briefly, total RNA was isolated from cells using TRIzol (Gibco-BRL) and 30 µg of total RNA was hybridized to <sup>32</sup>P-labeled probes for each indicated RNA message. Analysis was performed using a Storm Phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA). Results were normalized to the expression of GAPDH as an internal control.

## Western blot analysis

Protein extracts were prepared from cells using a protein lysis buffer containing 50 mM Tris-HCl, pH 7.5, 2.0 mM phenylmethylsulfonyl fluoride (PMSF), 5.0 mM iodoacetamide, 5.0 mM ethylene diamine tetra-acetic acid (EDTA), 150 mM NaCl, 0.5% nonylphenoxy polyethoxy ethanol (NP-40), and 0.5% nonanoyl-N-methylglucamide (Mega-9). Protein concentrations in the extracts were quantitated using bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). From each extract, 50 µg of total protein was separated on a 12% SDS-PAGE. After electrophoresis, proteins were transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked for 1 h at room temperature in 5% powdered milk dissolved in 1X Tween-20, Tris-buffered saline (TTBS) containing 14 mM Tris, 154 mM NaCl, and 0.1% Tween-20, pH adjusted to 7.5 with HCl. The membrane was incubated with the primary antibody for 16 h at 4°C. The antigen-antibody complexes were detected by using the appropriate secondary antibodies conjugated to horseradish peroxidase (Promega, Madison, WI, USA) at 1:2500 dilution. Membranes were developed using ECL+ (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) and films exposed for 5 or 30 min. Primary antibodies used were: mouse monoclonal anti-human Bcl-2 (C-2, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:500 dilution, rabbit polyclonal anti-human Bax (N-20, Santa Cruz Biotechnology) at 1:400 dilution, mouse monoclonal anti-androgen receptor (N-20, Santa Cruz Biotechnology) at 1:1000 dilution, goat polyclonal anti-human prostate specific antigen (C-19, Santa Cruz Biotechnology) at 1:2000 dilution, mouse monoclonal anti-human Bcl-xL(H-5, Santa Cruz Biotechnology) at 1:100 dilution, and mouse monoclonal anti-human  $\beta$ -actin (Sigma) at 1:1000 dilution.  $\beta$ -actin or a rabbit polyclonal anti-human PGK antibody at a 1:2000 dilution was included in the Western blot analysis as an internal control.

## PCR analysis

Bcl-2 message was characterized by PCR using the enhanced Avian RT-PCR kit (Sigma). Total RNA (5 µg) was added to each RT reaction using random nonamer primers. The cDNA products were amplified using Bcl-2 specific primers to give a 660 bp product. The forward primer (corresponding with nucleotides 1-20 of human Bcl-2 sequence) used was 5'-GTTGGCCCCCGTTACT-TTTC-3', and the reverse primer (corresponding to nucleotides 660-642 of the human Bcl-2 sequence) used was 5'-AACAGAGGCCGCGATGCTGGG-3'.

## Confocal microscopy

LNCaP cells were grown to 80% confluence on the surface of round glass cover slips in 12-well plates. The cells were washed with HBSS and blocked for 1 h in SuperBlock blocking buffer (Pierce, Rockford, IL, USA). Cells were then incubated with anti Bcl-2 primary antibody at 1:500 dilution for 1 h at room temperature. Cells were washed three times in 1 × phosphate buffered saline (PBS), and incubated with 1:10 000 dilution of Texas Red Anti-mouse

IgG secondary antibody (Vector Labs, Burlingame, CA, USA) for 1 h. Cells were washed three times in 1 × PBS, mounted onto glass slides using Vectashield mounting media (Vector Labs) and visualized by confocal microscope with a Carl Zeiss Model LSM 410 microscope.

## TUNEL assay

Cells were seeded onto round glass cover slips placed into 12-well plates and allowed to grow for 3 days. The cells were then washed once with HBSS and then treated with 500 µM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in RPMI 1640 or left untreated and fed with RPMI 1640 and incubated for 24 h. Apoptotic cells were determined using the TUNEL assay (Roche Molecular Biochemicals, Manneheim, Germany). Briefly, cells were rinsed once in 1 × PBS and fixed with 4% paraformaldehyde in 1 × PBS for 1 h at room temperature. Cells were then rinsed with 1 × PBS and endogenous peroxidase activity blocked with 3% H<sub>2</sub>O<sub>2</sub> in methanol for 10 min at room temperature. Cells were permeabilized with 0.1% triton × - 100 in 1.0% sodium citrate buffer for 2 min on ice. After three rinses in 1 × PBS, cells were incubated with terminal deoxynucleotidyl transferase (TUNEL) for 1 h at 37°C. Cells were then rinsed three times in 1 × PBS, and incubated with labeling solution for 30 min at 37°C. Cells were then rinsed three times in 1 × PBS, and incubated with diaminobenzamidine (DAB) in peroxide buffer for 2 min. The coverslips were then rinsed in Milli-Q H<sub>2</sub>O and mounted onto glass slides. Analysis of apoptotic cells was performed under a light microscope at 400 × magnification by counting positively stained nuclei.

## DNA fragmentation assay

Cells were treated with 500 nM paclitaxel for 24 h, 5 mM sodium butyrate for 72 h, or left untreated for the same durations. For DNA fragmentation analysis, cells were lysed at 48°C for 30 min in a DNA lysis buffer containing 20 mM EDTA, 5 mM Tris-HCl (pH 7.4), and 0.5% (v/v) Triton × - 100. Lysates were clarified by centrifugation at 15000 rpm in an Eppendorf microcentrifuge for 15 min and the supernatant was treated with RNase A (20 µg/ml) at 37°C for 30 min followed by treatment with proteinase K (20 µg/ml) for 30 min at 37°C. DNA fragments were precipitated with 95% ice-cold ethanol and pelleted by centrifugation for 15 min at 15 000 rpm in an Eppendorf microcentrifuge. DNA pellets were resuspended in Milli-Q water and subjected to electrophoresis on a 1.5% agarose gel. The gel was stained with ethidium bromide (0.5 µg/ml) and the DNA fragmentation was visualized using a Nucleovision Nucleotech Gel Expert (Hayward, CA) gel imaging workstation.

## Lactate dehydrogenase assay

Determination of necrosis was performed by measuring the presence of lactate dehydrogenase (LDH) in the cell culture media. Cells were seeded and allowed to grow to 80% confluence in T75 flasks, and then treated with 5.0 mM sodium butyrate or left untreated (control) for 72 h. LDH activity present in the media of treated and



control cells were determined using Cytotox 96 (Promega) kit. Briefly, 1.0 ml of media from each flask of treated and control cells were collected and centrifuged for 5 min at 14000 rpm at 4°C in an Eppendorf microcentrifuge. A volume of 50 µl of the centrifuged media was added in triplicate to a 96-well plate. To control for LDH present in serum contained in the media, 50 µl of serum-containing media was added in triplicate as a blank. For a positive control, 50 µl of cell lysate from the treated LNCaP cells were added in triplicate. Substrate mix (50 µl) was added to each sample and the plate incubated in darkness for 30 min at room temperature. Stop solution (50 µl) was added to each well and the absorbance at 490 nm was recorded on a Spectramax 190 plate reader (Molecular Devices).

## Results

### Characterization of the LNCaP progression model

We utilized the LNCaP prostate cancer cell line maintained under normal growth conditions as described in the Materials and methods section. At low passage numbers, LNCaP cells are androgen-responsive. By continued subculture under normal growth conditions, these cells become androgen-unresponsive at higher passages (Figure 1). Table 1 summarizes the passage numbers and designation of the LNCaP cells used in this study. We define LNCaP cell passages 20–50 as LNCaP-responsive (LNCaP-R), passages 50–95 as LNCaP-responsive fast growing (LNCaP-RF), and passages 95 and above as LNCaP-unresponsive (LNCaP-UR).

All representatives of the LNCaP model expressed the androgen receptor (Figure 2, panel A) and we observed a gradual decrease in expression of the prostate specific antigen as the cells progressed from the androgen-respon-

sive (LNCaP-R) to androgen-unresponsive (LNCaP-UR) state (Figure 2, panel B).

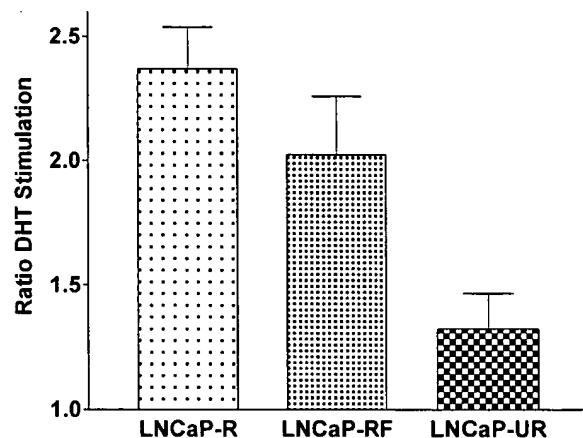
### The Bcl-2 family of apoptosis regulators is normal in the LNCaP model

We utilized the RNase protection assay to determine mRNA expression of Bcl-2 family of genes in LNCaP cells grown under normal conditions. Figure 3 shows the expression of various members of the Bcl-2 family normalized to GAPDH as a control. Both the anti-apoptotic (Bcl-2, Mcl-1, Bclx, and Bfl-1) and proapoptotic (Bax, Bik, and Bak) genes in the Bcl-2 family were expressed in the LNCaP-R, LNCaP-RF and LNCaP-UR cells. There were no significant differences in the expression of Bcl-2 family members in the three LNCaP cell types. We further confirmed Bcl-2 message expression in the LNCaP model by RT-PCR (Figure 4). All three LNCaP cell types in our model showed Bcl-2 message expression, which confirmed the results of the RNase protection assay (Figure 3) that Bcl-2 message is present in the all representatives of the LNCaP model.

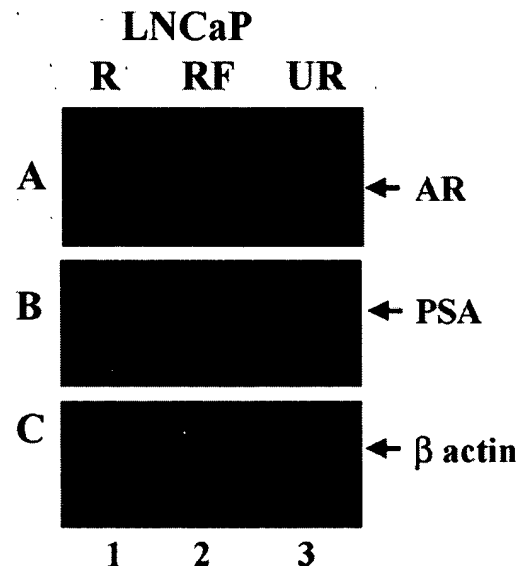
**Table 1** LNCaP progression model

Passage number	Designation
20–50	LNCaP-R
50–95	LNCaP-RF
95 and above	LNCaP-UR

LNCaP cells of increasing passage numbers were given the designations R (responsive), RF (responsive fast growing), and UR (fast growing unresponsive) according to their growth properties and responsiveness to androgen stimulated growth.



**Figure 1** Response of LNCaP cell types to androgen stimulation. Response of the LNCaP model to androgen stimulation was determined by treatment of cells with 10 nM DHT for 4 days and measurement of cell growth by MTT assay as described in the Materials and methods section. The data shown (mean ± s.d.) are from two independent experiments with each sample in triplicate.

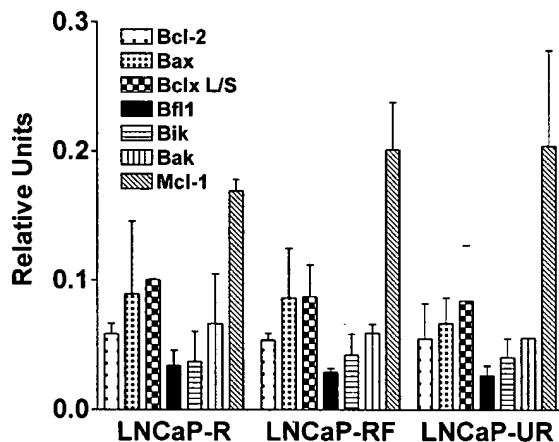


**Figure 2** Characterization of the LNCaP model. To characterize the LNCaP model used in this study, expression of the androgen receptor (panel A) and prostate specific antigen (panel B) was determined by Western blot analysis as described in the Materials and methods section. β-actin (panel C) was included as an internal control.

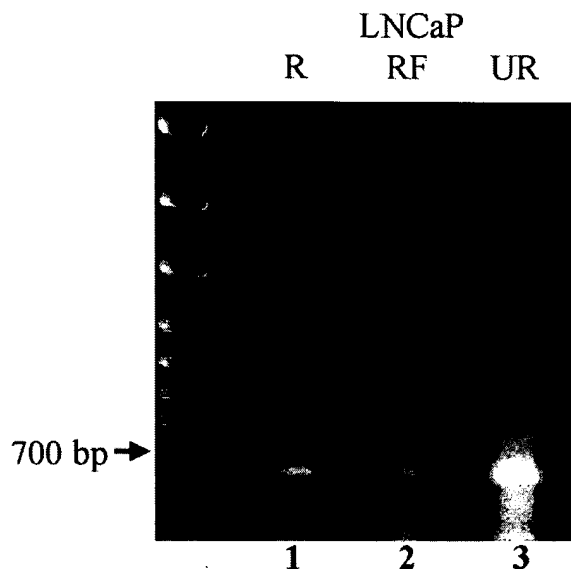
### Bcl-2 protein is undetectable in androgen-unresponsive cells

We utilized Western blotting to determine if the corresponding Bcl-2 protein expression was present in the LNCaP model. Two representative passages from each of the LNCaP model were used along with the androgen-responsive cell lines MDAPCa 2A and 2B (Figure 5). LNCaP-R and RF (Lanes 2–5) showed similar expression of Bcl-2. LNCaP-UR cells (lanes 6 and 7) had no expression of Bcl-2 protein either after a short exposure (panel A) or prolonged exposure (panel B) of the membrane to the X-ray film. MDAPCa 2B (lane 9) showed Bcl-2 protein

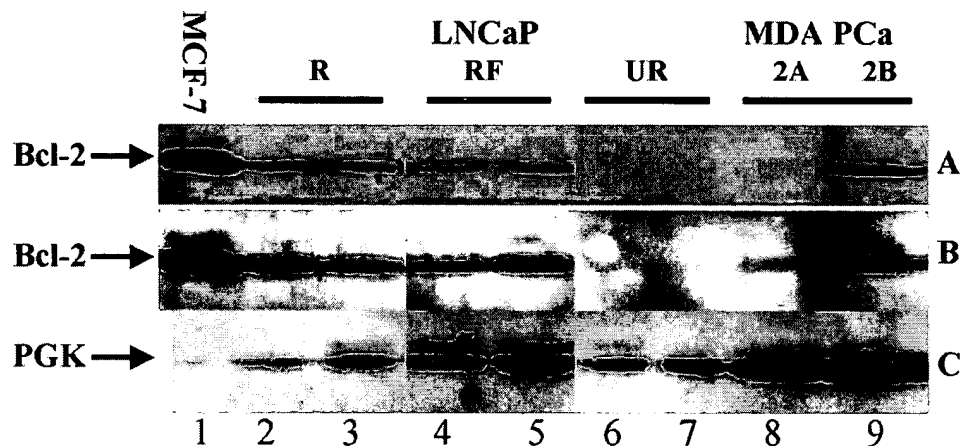
expression but only a faint amount of Bcl-2 was detectable in MDAPCa 2A cells (lane 8) after 30 min exposure of the membrane. This finding is consistent with Navone *et al.*<sup>14</sup> who showed that both MDAPCa 2A and 2B expressed Bcl-2. To normalize the protein levels of Bcl-2, we included an immunoblot against phosphoglycerate kinase (PGK) (panel C, Figure 5) as an internal control.



**Figure 3** mRNA expression of the Bcl-2 family is equal in the LNCaP model. The Bcl-2 family of apoptosis regulators mRNA expression was determined by RNase protection assay as described in the Materials and methods section. The data shown are the results of two independent experiments and are normalized to the expression levels of GADPH. The data shown (mean  $\pm$  s.d.) are from two independent experiments.



**Figure 4** RT-PCR confirms mRNA expression of Bcl-2 in the LNCaP model. RNA was isolated from cells using TRIzol reagent (Gibco-BRL) and reverse transcribed using Sigma Avian RT-PCR kit (Sigma-Aldrich) as described in the Materials and methods section. The PCR product of Bcl-2 was amplified using primers that targeted the 5' end of Bcl-2 mRNA and gave the expected 660 bp product.

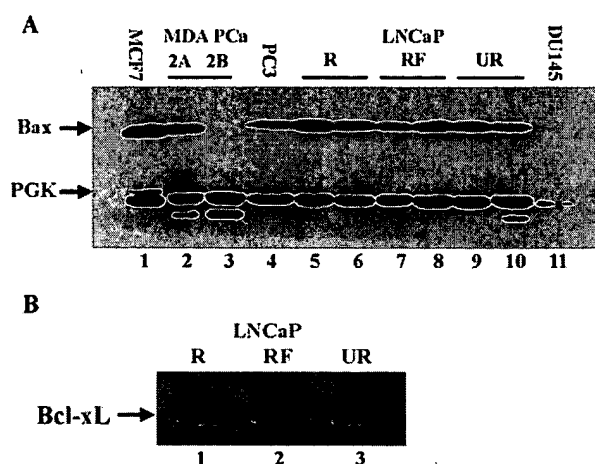


**Figure 5** Comparison of Bcl-2 protein expression in LNCaP and MDA PCa 2A and 2B. Bcl-2 protein expression was measured in the LNCaP model (R, RF and UR) and in the MDA PCa 2A and 2B androgen-responsive cell lines. Protein extracts from MCF-7 (lane 1, 20  $\mu$ g), various LNCaP cell types (50  $\mu$ g protein, lanes 2–7) and MDAPCa cells (50  $\mu$ g protein, lanes 8 and 9) were subjected to SDS-PAGE and immunoblotting as described in the Materials and methods section. The membrane was exposed to X-ray film for 5 min (panel A). Prolonged exposure of the membrane for an additional 30 min (panel B) did not result in the appearance of any additional bands. Immunoblot analysis of phosphoglycerate kinase (panel C) was included as an internal control.

In each analysis, we also included the MCF-7 breast carcinoma cell line (Figure 5, Lane 1) as a positive control since these cells endogenously express high levels of Bcl-2 protein.

#### Bax and Bcl-xL protein levels are unchanged in the LNCaP model

Bax is an important pro-apoptotic protein thought to heterodimerize with Bcl-2 and thus promote apoptosis.



**Figure 6** Bax and Bcl-xL protein expression in androgen-responsive and androgen-unresponsive cells. Bax protein expression (panel A, top) was measured by immunoblot analysis in MCF 7 (lane 1, 20 µg protein), MDA PCa 2A and 2B (50 µg protein, lanes 2, 3), PC3 (50 µg protein, lane 4), LNCaP model (50 µg protein, lanes 5-10) and DU145 (50 µg protein, lane 11) cells. Protein was extracted and analyzed as described in the Materials and methods section. Analysis of PGK (panel A, bottom) was included as an internal control. The membrane was exposed for 5 min and overexposure for 30 min did not result in the appearance of any additional bands (data not shown). Immunoblot analysis of Bcl-xL expression (panel B) was analyzed in LNCaP-R, LNCaP-RF and LNCaP-UR cells as described in the Materials and methods section using anti-Bcl-xL antibody.

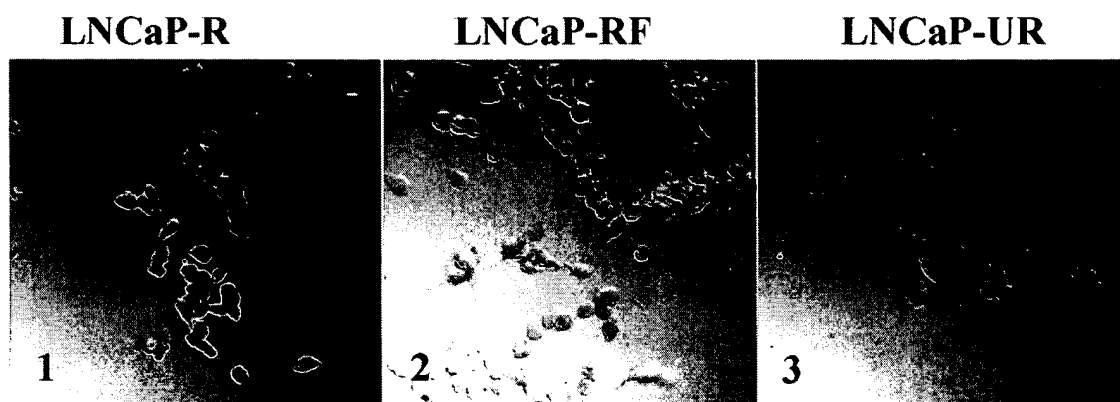
Since it is thought that the ratio of Bcl-2 to Bax determines the susceptibility of cells to undergo apoptosis,<sup>7</sup> we examined Bax protein levels in the LNCaP model (Figure 6, panel A). All representatives of the LNCaP model (lanes 5-10) showed Bax expression at similar levels. Bax expression was seen in the MDAPCa 2A cell line (lane 2), but not the MDAPCa 2B cell line (lane 3) which is consistent with previously published literature.<sup>14</sup> PC3 cells (lane 4) exhibited Bax expression while it was absent in the DU145 cell line (lane 11) which is consistent with previous published observations.<sup>7,15</sup> Immunoblot analysis of Bcl-xL (Figure 6, panel B) showed no changes in the Bcl-xL levels in the LNCaP model.

#### Confocal microscopy shows a basal level of Bcl-2 protein expression in androgen-unresponsive cells

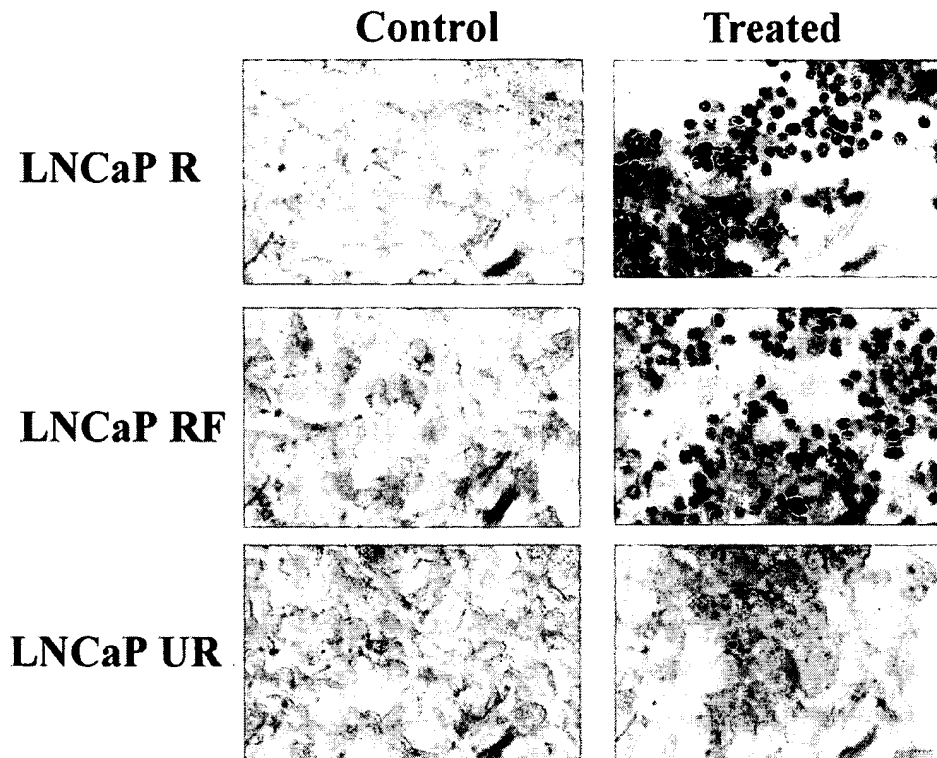
LNCaP cells were grown on glass cover slips and subjected to immunocytochemistry to determine the cellular expression of Bcl-2. Figure 7 shows LNCaP cells stained for Bcl-2 and visualized by confocal microscopy. LNCaP-R cells (panel 1) show a high level of staining for Bcl-2, while a lesser degree of staining for Bcl-2 was seen in LNCaP-RF cells (panel 2) when compared to LNCaP-R cells. LNCaP-UR cells (panel 3) show a low level of Bcl-2 staining suggesting that expression of Bcl-2 in these cells is present, but at a level below detection by Western blot analysis.

#### Androgen-unresponsive cells are resistant to apoptosis induced by oxidative stress but not by treatment with paclitaxel or sodium butyrate

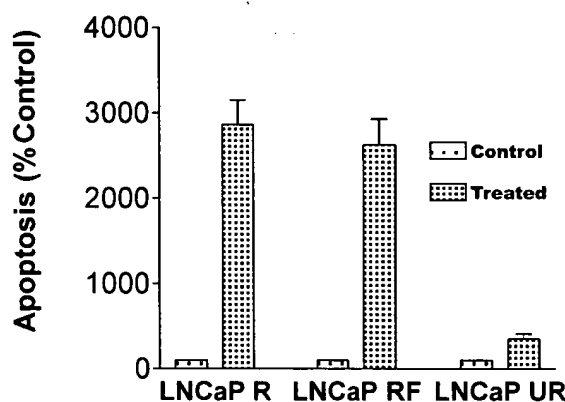
In order to test whether changes in Bcl-2 levels in the LNCaP model affects the sensitivity of these cells to various apoptosis inducers, cells were treated with H<sub>2</sub>O<sub>2</sub> and examined for DNA fragmentation using the TUNEL assay. LNCaP-R, RF and UR were treated for 24 h in 500 µM H<sub>2</sub>O<sub>2</sub> to introduce oxidative damage and induce apoptosis. LNCaP-R (Figure 8) shows a significant



**Figure 7** Confocal microscopy shows a low basal level of Bcl-2 protein in androgen-unresponsive cells. LNCaP-R (panel A), LNCaP-RF (panel B) and LNCaP-UR (panel C) cells were probed for endogenous Bcl-2 protein expression in situ. Cells were grown for 3 days on glass coverslips placed into 12-well plates. Cells were processed as described in the Materials and methods section. Confocal analysis was performed using Carl Zeiss Model LSM410 microscope (×200 magnification).



**Figure 8** Photomicrographs of LNCaP model treated with  $H_2O_2$ . Representative photomicrographs of TUNEL stained cells treated with 500  $\mu M$   $H_2O_2$  or left untreated for 24 h then fixed in 4% paraformaldehyde and processed for the TUNEL assay as described in the Materials and methods section. TUNEL positive staining, apparent as darkly stained nuclei, represent apoptotic cells ( $\times 200$  magnification).



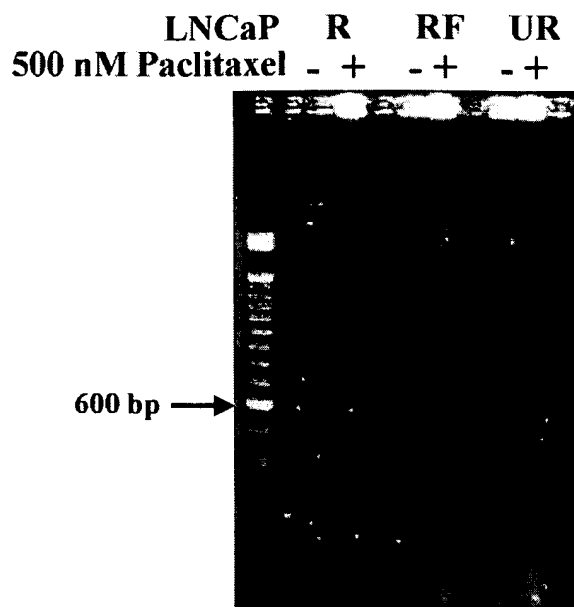
**Figure 9** Androgen-unresponsive cells are resistant to apoptosis induced by oxidative stress. Cells were treated with 500  $\mu M$   $H_2O_2$  or left untreated and processed for the TUNEL assay as described under Materials and methods. Quantification of apoptotic cells was performed by counting positively stained cells under a light microscope. Approximately 160 cells (per  $40\times$  field) were counted for each sample in quadruple. Data are means  $\pm$  s.e.m.

amount of apoptosis as do the LNCaP-RF cells. However, LNCaP-UR cells show little TUNEL positive staining indicating that these cells are resistant to apoptosis

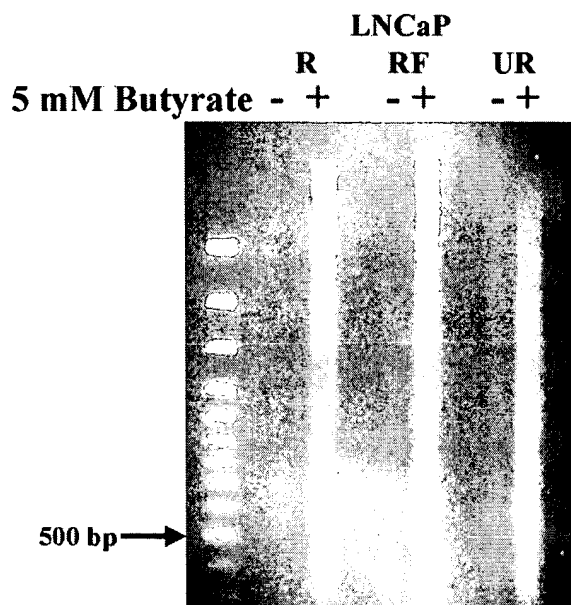
induced by oxidative stress. Figure 9 shows quantitation of the TUNEL data. The data indicates the relative resistance of LNCaP-UR cells to undergo apoptosis induced by  $H_2O_2$ . In these cells, some changes or modifications in the apoptotic machinery have taken place to lend resistance to oxidative stress.

LNCaP-R, RF and UR were induced to undergo apoptosis by treatment with 500 nM paclitaxel for 24 h. Paclitaxel is a microtubule active drug and functions by disruption of the cytoskeleton and is also thought to induce apoptosis by phosphorylation and subsequent degradation of Bcl-2 in prostate cancer cells.<sup>16,17</sup> Given the loss of expression of Bcl-2 in the LNCaP-UR cells, we sought to determine if paclitaxel could induce apoptosis irrespective of Bcl-2 status. Figure 10 shows the results of a DNA ladder experiment and shows DNA fragmentation in all treated lanes suggesting that LNCaP-R, RF and UR are equally sensitive to this drug.

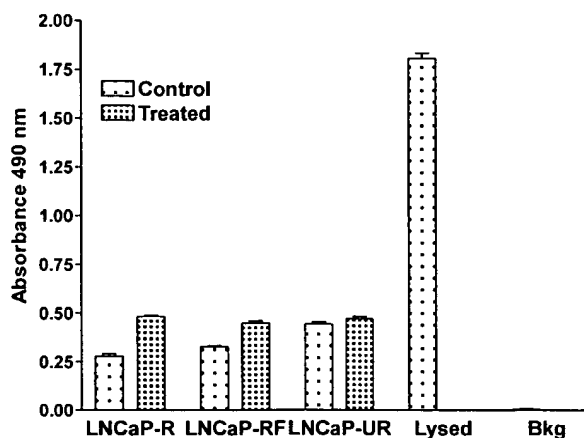
We also induced apoptosis in the LNCaP model with sodium butyrate. Sodium butyrate acts to disrupt cellular function and induce apoptosis by a number of mechanisms, one of which is histone deacetylation inhibition that alters gene expression. We found that treatment of the LNCaP model for 72 h with 5 mM of sodium butyrate was sufficient to induce apoptosis as evidenced by DNA fragmentation analysis (Figure 11). To distinguish between cell death by apoptosis or necrosis, we measured LDH activity as an indicator of necrosis (Figure 12).



**Figure 10** Both androgen-responsive and androgen-unresponsive cells are sensitive to paclitaxel. Cells were treated with 500 nM paclitaxel and control cells with vehicle alone (0.1% ethanol). After treatment for 24 h, cells were harvested and DNA was extracted as described in the Materials and methods section. DNA was visualized by electrophoresis in a 1.5% agarose gel stained with 0.5 µg/ml ethidium bromide.



**Figure 11** Both androgen-responsive and androgen-unresponsive cells are sensitive to sodium butyrate. LNCaP Cells were treated with 5.0 mM sodium butyrate for 72 h or left untreated. After 72 h, treated and control cells were harvested and DNA was extracted as described in the Materials and methods section. DNA fragments were separated and visualized by electrophoresis in a 1.5% agarose gel stained with 0.5 µg/ml ethidium bromide.



**Figure 12** Cell death in sodium butyrate treated and control LNCaP cells is not due to necrosis. Cells were treated with 5.0 mM sodium butyrate or left untreated for 72 h then harvested and processed for LDH determination as described in the Materials and methods section. Absorbance was read at 490 nm and data shown as absorbance values normalized to background. Each datum point represents the mean  $\pm$  s.e.m. from triplicate observations from two separate experiments.

Exposure of cells to sodium butyrate did not result in increased LDH release into the media indicating that necrosis was not involved in sodium butyrate induced cell death in the LNCaP model. Results shown in Figures 11 and 12 suggest that sodium butyrate can induce apoptosis in the LNCaP model irrespective of Bcl-2 status.

## Discussion

The purpose of this study was to measure changes in the Bcl-2 family of apoptosis effectors during the progression of prostate cancer from an androgen-responsive to an androgen-unresponsive state. Our focus was on Bcl-2 since this anti-apoptotic protein has been demonstrated to play an important role in androgen-insensitive prostate cancer.<sup>4</sup> The LNCaP prostate carcinoma cell model used in this study offered the advantage of having a similar genetic background to compare the expression of Bcl-2 in the progression of prostate cancer to an androgen-unresponsive state.

Message expression of Bcl-2 was present in all representatives of the LNCaP model (Figure 3). RT-PCR confirmed that message expression was present (Figure 4) which led us to further examine Bcl-2 protein levels. We expected that Bcl-2 protein expression would increase as cells became androgen-unresponsive. However, we failed to observe an increase in Bcl-2 protein expression as the LNCaP cells became androgen-unresponsive (Figure 5), but observed a dramatic decrease in Bcl-2 protein levels. This finding is consistent with the observation that in many prostate cancer tissue specimens, Bcl-2 staining is negative despite a high Gleason grade and stage.<sup>6</sup>

The finding that Bcl-2 protein expression was lost in the androgen-unresponsive LNCaP cells suggest a number of possibilities, one of which is that Bcl-2 may

not be the determining factor in the progression of prostate cancer to androgen-unresponsiveness and the concomitant resistance to apoptosis. The notion that Bcl-2 is not the determining factor in apoptosis sensitivity in the LNCaP model was confirmed by subjecting the cells to oxidative stress to undergo apoptosis. We found that the LNCaP-UR cells were highly resistant despite the apparent loss of expression of Bcl-2 protein. Another possibility is that Bcl-2 may be rapidly degraded in our LNCaP-UR cells. Several studies have shown that modification of the N-terminal region of Bcl-2 targets this protein for rapid degradation. Breitschopf *et al.*<sup>18</sup> showed that dephosphorylation of serine 87 led to the proteasome-mediated degradation of Bcl-2 in HUVEC cells. Srivastava *et al.*<sup>19</sup> showed that a deletion of the loop region of Bcl-2, which removes a segment of the N-terminal region of the Bcl-2 protein abrogates the ability of cells expressing this mutant protein to undergo paclitaxel induced apoptosis. Whether or not our LNCaP-UR cells have these N-terminal modifications of Bcl-2 has yet to be determined.

The proapoptotic Bax protein is thought to heterodimerize with Bcl-2 and affect the ability of Bcl-2 to block apoptosis. It is thought that increased protein expression of Bax favors apoptosis and decreased expression of Bax favors survival.<sup>20</sup> Bax protein was equally expressed in all representatives of the LNCaP model (Figure 7). The loss of Bcl-2 in the LNCaP-UR cells did not correspond to any change in Bax protein expression. Therefore if an increase of Bax protein targets cells for apoptosis, our LNCaP-UR cells would have been sensitive to oxidative damage. However, this was not the case (Figures 8 and 9). This finding suggests that the Bcl-2 to Bax protein ratio may not be the determinant of apoptosis sensitivity in LNCaP cells. Bcl-2 is the founding member of a growing family of cell survival and death arbiters and numerous examples of antiapoptotic role of Bcl-2 in cancer have been demonstrated.<sup>21</sup> A number of other Bcl-2 family members are yet to be characterized and their potential role in prostate cancer has yet to be elucidated. Bcl-xL is an antiapoptotic member of the Bcl-2 family and was shown to desensitize LNCaP and PC3 cells to chemotherapeutic agents when over-expressed, and sensitized these cell lines to chemotherapeutic agents when down-regulated with antisense oligonucleotides.<sup>22</sup> In our model, we did not observe any changes in the level of Bcl-xL protein (Figure 6B) in LNCaP-R, LNCaP-RF or LNCaP-UR cells.

Interestingly, when the LNCaP-model was treated with paclitaxel, all of the LNCaP cell types were equally sensitive to this drug (Figure 10). A number of studies report that one mechanism of action of paclitaxel is phosphorylation of Bcl-2, which targets this protein for proteasome-mediated degradation.<sup>18,19</sup> Since LNCaP-UR cells do not express Bcl-2, the mechanism of paclitaxel-induced apoptosis in these cells may be a result of its microtubule disrupting action or by a yet unidentified action of the drug. Treatment of the LNCaP model with sodium butyrate showed that both our androgen-responsive and androgen-unresponsive cells were equally sensitive to the induction of apoptosis with little or no cell death by necrosis (Figures 11 and 12). These data suggest that androgen-unresponsive cells can be induced to undergo apoptosis by similar mechanisms as their androgen-responsive counterparts.

## Acknowledgements

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# EXHIBIT B

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# Molecular Cloning and Characterization of *STAMP1*, a Highly Prostate-specific Six Transmembrane Protein that Is Overexpressed in Prostate Cancer\*

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We have identified a novel gene, six transmembrane protein of prostate 1 (*STAMP1*), which is largely specific to prostate for expression and is predicted to code for a 490-amino acid six transmembrane protein. Using a form of *STAMP1* labeled with green fluorescent protein in quantitative time-lapse and immunofluorescence confocal microscopy, we show that *STAMP1* is localized to the Golgi complex, predominantly to the *trans*-Golgi network, and to the plasma membrane. *STAMP1* also localizes to vesicular tubular structures in the cytosol and colocalizes with the early endosome antigen 1 (EEA1), suggesting that it may be involved in the secretory/endocytic pathways. *STAMP1* is highly expressed in the androgen-sensitive, androgen receptor-positive prostate cancer cell line LNCaP, but not in androgen receptor-negative prostate cancer cell lines PC-3 and DU145. Furthermore, *STAMP1* expression is significantly lower in the androgen-dependent human prostate xenograft CWR22 compared with the relapsed derivative CWR22R, suggesting that its expression may be deregulated during prostate cancer progression. Consistent with this notion, *in situ* analysis of human prostate cancer specimens indicated that *STAMP1* is expressed exclusively in the epithelial cells of the prostate and its expression is significantly increased in prostate tumors compared with normal glands. Taken together, these data suggest that *STAMP1* may have an important role in the normal prostate cell as well as in prostate cancer progression.

The prostate gland is a major secretory organ whose precise function is still not known (1). Through secretions into the male ejaculate, it is thought that the prostate protects the lower urinary tract from infection and increases fertility. Despite the unknown specific function, the prostate is the most common site of neoplastic transformation in men. Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer mortality in men other than skin cancer (2). In the

initial stages, prostate cancer is dependent on androgens for growth, which is the basis for androgen ablation therapy (3). However, in most cases, prostate cancer progresses to an androgen-independent phenotype for which there is no effective therapy available at present (for reviews, see Refs. 4 and 5).

Currently, there is limited information regarding the molecular details of normal prostate function as well as prostate cancer initiation and progression. Several independent approaches resulted in the identification of a few highly prostate-enriched genes that may have unique roles in these processes. The first such gene discovered was prostate-specific antigen (PSA)<sup>1</sup> (for a review, see Ref. 6), which is currently used as a diagnostic tool and also as a marker for the progression of prostate cancer, albeit with significant limitations (7, 8). More recently, several additional prostate-enriched genes were identified including prostate-specific membrane antigen (PSMA) (9), prostate carcinoma tumor antigen 1 (*PCTA-1*) (10), *NKX3.1* (11, 12), prostate stem cell antigen (*PSCA*) (13), *DD3* (14), and *PCGEM1* (15). Research on these genes and their products is likely to provide detailed information on normal and hyperplastic prostate biology as well as improving disease diagnosis, prognosis, and therapy. However, these goals have not yet been realized.

While searching for genes that are differentially expressed during early stages of prostate cancer (16), we have cloned a novel gene, named six transmembrane protein of prostate 1 (*STAMP1*). *STAMP1* is highly specific to prostate for expression. GFP-tagged *STAMP1* in immunocytochemistry and time-lapse imaging studies indicate that *STAMP1* may have a role in endocytic and/or secretory trafficking pathways. Furthermore, *STAMP1* expression is increased in androgen-independent prostate cancer xenografts compared with their androgen-dependent counterparts as well as in prostate tumors compared with normal prostate. These data suggest that *STAMP1* may have a key role in both normal prostate physiology and the progression of prostate cancer.

## MATERIALS AND METHODS

**Cell Culture**—LNCaP, PC-3, and DU145 cells were routinely maintained and treated as described previously (16).

**Xenograft Studies**—Transplantation, growth, and harvesting of tumors from mice bearing the CWR22 and CWR22R xenografts were as previously described (17, 18).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY008445.

¶ These authors contributed equally to this work.

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<sup>1</sup> The abbreviations used are: PSA, prostate-specific antigen; *STAMP1*, six transmembrane protein of prostate; GFP, green fluorescent protein; RACE, rapid amplification of cDNA ends; UTR, untranslated region; ORF, open reading frame; TIAP, tumor necrosis factor  $\alpha$ -induced adipose-related protein; ManII, mannosidase II; VTS, vesicular tubular structures; AR, androgen receptor; STEAP, six transmembrane epithelial antigen of the prostate.

**Cloning and Plasmid Construction**—A 262-bp cDNA fragment was originally obtained from a screen of a prostate-specific library (16) and termed L74. RACE (5'-rapid amplification of cDNA ends) was performed (oligonucleotide sequences available upon request) using the Marathon-Ready cDNA that was prepared from normal prostate tissue (CLONTECH) and/or SMART-RACE LNCaP cDNA library (CLONTECH) that was generated according to the manufacturer's recommendations. RACE products were cloned into pCRII-TOPO (Invitrogen), and positive clones were confirmed by Southern analysis and sequenced. In parallel, a  $\lambda$ gt10 cDNA library made from a pool of normal human prostates (CLONTECH) was screened by established procedures (19) to obtain additional clones. Overlapping clones were used to deduce the full-length STAMP1 cDNA sequence.

The full-length STAMP1 ORF was amplified by using primers centered around the start and stop codons (sequences available upon request) and fused in-frame to the C terminus of GFP using the vector pcDNA3.1-NT-GFP-TOPO (Invitrogen) to generate GFP-STAMP1.

**Protein Sequence Analysis**—Primary sequence analysis for STAMP1 was performed in BLAST ([www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)). Secondary protein structure predictions were performed using the web tools SMART ([smart.embl-heidelberg.de/](http://smart.embl-heidelberg.de/)), SOSUI ([sosui.proteome.bio.tuat.ac.jp/sosui/frame0E.html](http://sosui.proteome.bio.tuat.ac.jp/sosui/frame0E.html)), and PSORT ([psort.nibb.ac.jp/](http://psort.nibb.ac.jp/)).

**Northern Analysis**—Total RNA was prepared by the single step guanidine thiocyanate procedure and used in Northern analysis (19). 15  $\mu$ g of total RNA were used per lane. Probes were generated by random priming and had a specific activity of  $>3 \times 10^6$  dpm/ $\mu$ g. A cDNA fragment of STAMP1 spanning 145–2202 bp was used as a probe. Bands were visualized and quantitated by phosphorimaging analysis (Amersham Biosciences).

**Confocal Microscopy and Live Cell Imaging**—COS-1 cells were transfected by electroporation using a BTX square-wave pulser at 150 V, 1 ms duration. Cells were grown either on coverslips placed in 6-well tissue culture plates for indirect immunofluorescence or on Lab-Tek Chambered Coverglass (Nalge Nunc International) for live-cell microscopy. Transiently transfected cells were observed 18 h after transfection by a Leica TCS-SP confocal microscope using a 488-nm argon laser line. All live-cell experiments were done at 37 °C.

**Indirect Immunofluorescence**—Indirect immunofluorescence was carried out as previously described (20). The following antibodies were used: anti- $\beta$ -coat protein ( $\beta$ -COP) antiserum (kindly provided by J. Lippincott-Schwartz), anti-mannosidase II (kindly provided by T. Misteli), anti-TGN46 (Serotec, kindly provided by J. S. Bonifacino), and anti-EEA1 (Affinity Biotechnologies). Texas Red-conjugated secondary antibodies specific for mouse and rabbit were purchased from ICN Biomedicals (Costa Mesa, CA).

**In Situ Hybridization**—The STAMP1 riboprobe was made on a STAMP1 cDNA fragment corresponding to nucleotides 1339–2074 of STAMP1 cDNA. In parallel and as a negative control, a sense probe was generated corresponding to nucleotides 171–830 of STAMP1 and used in *in situ* analysis that did not give any specific staining as shown. The procedure was essentially as described previously (51).

## RESULTS

**Isolation and Characterization of the STAMP1 Gene and mRNA**—While searching for prostate-specific genes that are regulated in the early stages of prostate cancer (16), we cloned a 262-bp novel cDNA fragment, termed L74, which did not show any sequence similarity to the sequences in GenBank<sup>TM</sup>. By screening a normal prostate cDNA library and by 5'- and 3'-RACE analysis, we obtained the full-length cDNA for L74. Because computer-aided secondary structure prediction of the deduced amino acid sequence of L74 suggested the presence of a six transmembrane domain in its C-terminal half, we named L74 STAMP1.

When the full-length STAMP1 cDNA was used in BLAST analysis we found that it completely matched a BAC clone (GenBank<sup>TM</sup> accession no. AC002064) except for a 313-bp repetitive unit in the 3'-UTR region (data not shown), thereby identifying it as the STAMP1 gene and localizing it to Chr7q21. The repetitive region is likely to be a cloning or sequencing artifact of the BAC clone. Computational exon/intron junction analysis and alignment of the full-length cDNA sequence with the BAC clone revealed that the STAMP1 gene is composed of six exons and five introns (data not shown). The STAMP1 gene

spans around 26 kb, which is in part due to the extremely large size of intron 2 (12713 bp). There are three different predicted promoters within 4 kb upstream of the STAMP1 initiation codon (data not shown), none of which has any significant TATA or CAAT box consensus sequences, indicating that STAMP1 is transcribed from a TATA-less promoter.

The STAMP1 cDNA has a predicted 5'-UTR of ~1 kb (deduced by RACE analysis, data not shown) and an unusually long 3'-UTR of ~4 kb that comprises ~77% of the total cDNA sequence (data not shown). The ORF starts within the third exon and is predicted to encode a 490 amino acid protein (Fig. 1A). A search for known protein motifs identified six predicted transmembrane domains in the C-terminal half of STAMP1 starting at Phe-209 (Fig. 1B).

**STAMP1 Belongs to a New Subfamily of Six Transmembrane Domain Proteins**—BLAST analysis in GenBank<sup>TM</sup> with the predicted STAMP1 amino acid sequence identified two recently discovered proteins that showed significant similarity to STAMP1 over the entire ORF: the rat protein pHyde (21), which, when overexpressed, can cause apoptosis in prostate cancer cells and the tumor necrosis factor  $\alpha$ -induced adipose-related protein (TIARP) (22), a mouse protein which may have a role in adipocyte differentiation. TIARP and pHyde are 43 and 51% identical, respectively, to STAMP1 at the amino acid level. In addition, there was significant similarity in the C-terminal half of STAMP1 to STEAP, a recently discovered human cell membrane protein enriched in prostate for expression (23). An alignment of these sequences is shown in Fig. 1B. These data suggest that STAMP1, TIARP, and pHyde are structurally, and possibly also functionally, related proteins, and they therefore form a small six transmembrane protein subfamily that does not include STEAP.

**STAMP1 Is Highly Enriched to Prostate for Expression**—We next determined the expression profile of STAMP1 in various human tissues by Northern analysis in which a multiple tissue Northern blot was hybridized to the STAMP1 probe. As shown in Fig. 2A, STAMP1 hybridized to a major mRNA species of 6.5 kb and three minor mRNA species of 2.2, 4.0, and 4.5 kb in the normal prostate tissue. There was 15–20-fold lower mRNA expression of the 6.5-kb band in the heart, brain, kidney, pancreas, and ovary but not in other tissues. In contrast, the three lower molecular weight species, which are likely to be encoded by alternatively spliced forms of STAMP1,<sup>2</sup> were only detectable in the prostate. Hybridization with a glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA probe resulted in approximately similar signals in all lanes except for the heart and skeletal muscle where G3PDH is known to be more abundant compared with other tissues. These data show that STAMP1 is highly specific to prostate and that it may have isoforms that are restricted to prostate for expression.

**Characterization of STAMP1 Expression in Prostate Cancer Cell Lines and Xenografts**—Because androgen is a major hormonal stimulus for the normal prostate gland and for early stage prostate cancer (3), we assessed the possible androgen regulation of STAMP1 by Northern analysis in the androgen responsive prostate cancer cell line LNCaP (24). Cells were either left untreated or treated with the synthetic androgen R1881 for 24 h and harvested, and total RNA was isolated and used in Northern analysis with STAMP1 cDNA as probe. As shown in Fig. 2B, STAMP1 displayed similar expression levels in untreated and R1881-treated LNCaP cells. In contrast, the mRNA accumulation of the androgen-regulated gene NKX3.1 dramatically increased upon androgen stimulation in a time-

<sup>2</sup> K. Korkmaz, C. G. Korkmaz, and F. Saatcioglu, unpublished results.

A

MESISMMGSPKSLSETCLPNGINGIKDARKVTGVIGSGDFAKS  
 LTIRLIRCGYHVVISGRNPKFASEFFPHVVDVTHEDALTKTNI  
 IFVAIHREHYTSLWDLRHLVVGKILIDVSNMNRINQYPSNAEY  
 LASLFPDSLIVKGFNVVSAWALQGPKASRQVYICSNNIQARQ  
 QVIELARQLNFIPIIDLGLSLSSAREIENLPLRLFTFWRGFVVVAI  
 SLATFFFLYSFVRDVIHPYARNQOSDFYKIPIEIVNKTLPVIAI  
 TLLSLVYLAGLLAAAYQLYYGTYRRFPFWLETWLQCRKQLGLL  
 SFFFAMVHVAYSLLCLPMRRSERYLFNLMAVQQVHANIENSWNEE  
 EVWRIEMYISFGIMSLGLLSLLAVTSIPSVSNALNWREFSFIQS  
 TLGYVALLISTFHVLIYGWKRAFEERYRFTPPNFVLALVLP  
 IVILGKIILFLPCISRKLRKIKKGWEKSQFLEEGIGGTIPHVSP  
 ERTVTM

B

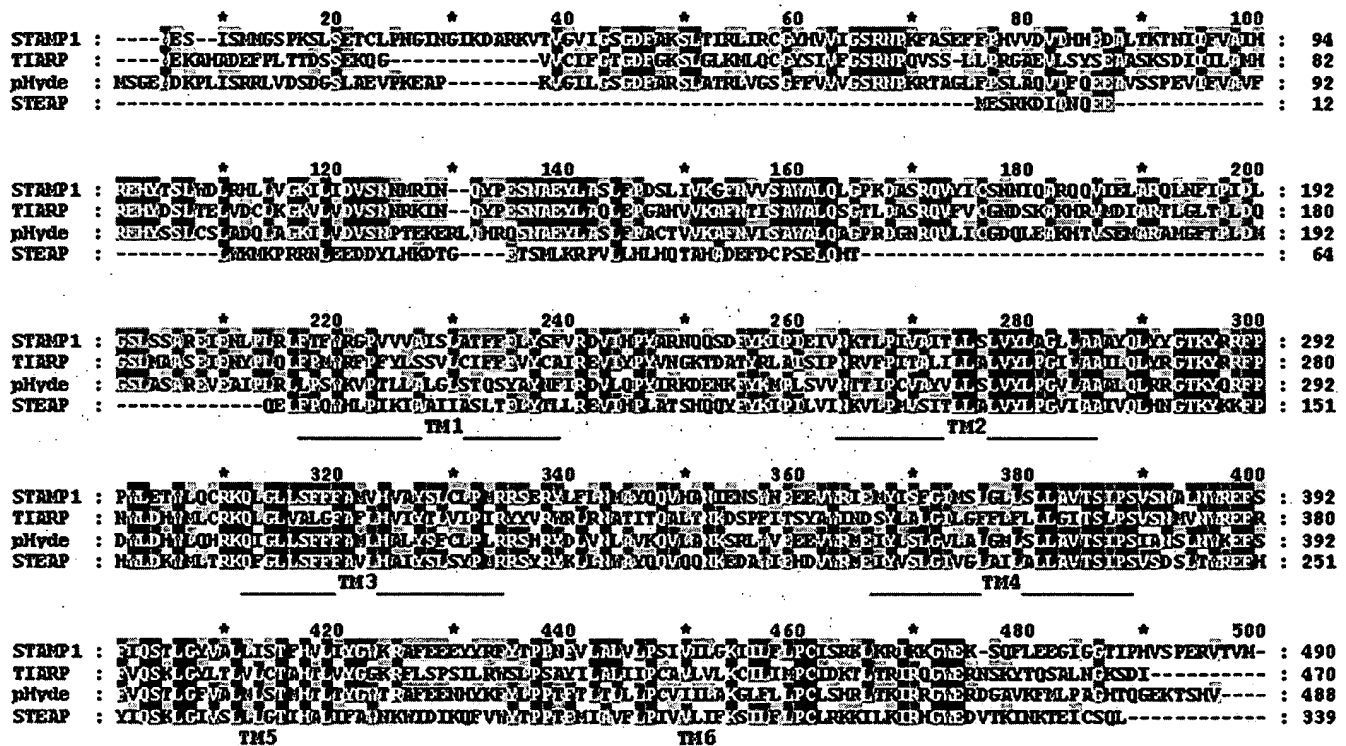


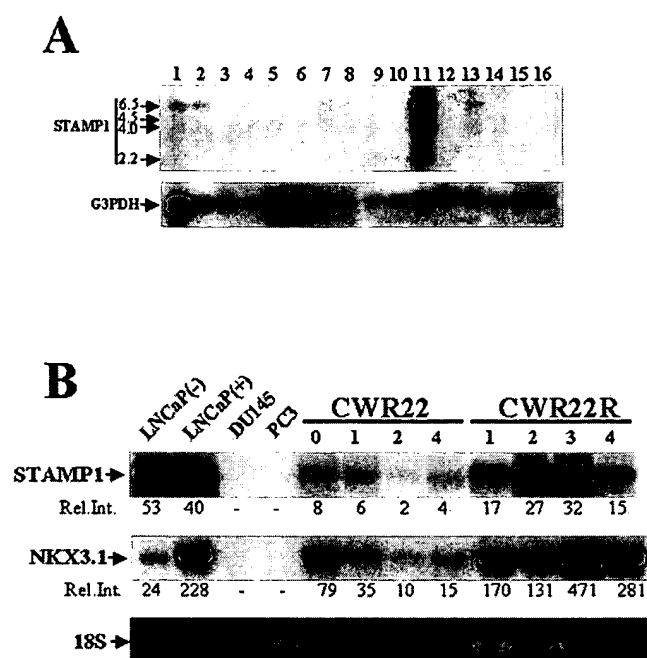
FIG. 1. Cloning of the *STAMP1* cDNA and sequence similarity to a subfamily of six transmembrane proteins. A, the full-length predicted open reading frame of *STAMP1* (GenBank<sup>TM</sup> AY008445). The predicted amino acid sequence of *STAMP1* is shown, and the locations of the predicted transmembrane domains are highlighted (TM 1-6). B, BLAST analysis identified TIARP (GenBank<sup>TM</sup> NP473439), pHyde (GenBank<sup>TM</sup> AAK00361.1), and STEAP (GenBank<sup>TM</sup> AF186249) as closely related recently identified proteins. The multiple sequence alignment obtained by Clustal and GenDoc programs is shown. Completely conserved residues are shaded in blue. Residues that are conserved in two or three of the sequences are shaded light and dark gray, respectively.

dependent manner, as expected (11, 25), reaching ~10-fold higher levels by 24 h. Time course analysis of androgen treatment did not result in significant differences in *STAMP1* expression.<sup>2</sup> These data suggested that *STAMP1* expression is not significantly regulated by androgens in LNCaP cells.

We also assessed the possible regulation of *STAMP1* expression in an *in vivo* setting using the recently developed androgen-dependent xenograft model CWR22, which is derived from a primary human prostate tumor (17). Because it is androgen-dependent for growth, the CWR22 tumors in nude mice display marked regression upon castration and may in fact regress completely (17). CWR22 xenografts were grown in nude mice in the presence of a sustained-release testosterone pellet (17). After the tumors had grown, mice were castrated, the testosterone pellets were removed, and the regressing tumors were collected at 1, 2, or 4 weeks postcastration. Total RNA was

prepared from these tumor samples and used in Northern analysis. As shown in Fig. 2B, similar to that observed in LNCaP cells, *STAMP1* mRNA accumulation in the CWR22 tumors showed no significant change upon castration and was not significantly affected by the presence of androgens. In contrast, the mRNA accumulation of the androgen-regulated gene *NKX3.1* was dramatically decreased upon castration, dropping to ~4% of precastrate levels by 4 weeks postcastration. These results are consistent with the findings in LNCaP cells and suggest that *STAMP1* expression is not significantly regulated by androgens in prostate cancer cells. Interestingly, *STAMP1* expression was substantially lower in the CWR22 tumors compared with LNCaP cells.

We also analyzed the expression profile of *STAMP1* in the androgen receptor-negative and therefore androgen-independent prostate cancer cell lines PC3 and DU145, as well as in



**Fig. 2. Characterization of STAMP1 expression.** A, a multiple tissue Northern blot (CLONTECH) was probed with STAMP1 or G3PDH cDNA. The stronger hybridization that is observed with G3PDH in the heart and skeletal muscle samples is due to its higher expression in these tissues. The lanes represent: heart (lane 1), brain (lane 2), placenta (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), pancreas (lane 8), spleen (lane 9), thymus (lane 10), prostate (lane 11), testis (lane 12), ovary (lane 13), small intestine (lane 14), colon (lane 15), and peripheral blood leukocyte (lane 16). The location of the full-length 6.5-kb mRNA, as well as the lower molecular weight STAMP1 species, are indicated by arrows to the left of the figure. B, LNCaP cells were either left untreated or treated with the synthetic androgen R1881 ( $10^{-8}$  M) for 24 h as indicated. Similarly, PC3 and DU145 cells were cultured but were left untreated. Total RNA was isolated and used in Northern analysis with STAMP1 as the probe. The same membrane was also probed for the androgen-dependent gene PSA. Relative induction of mRNA accumulation is indicated at the bottom of the lanes determined by phosphorimaging analysis (Amersham Biosciences). The CWR22 xenograft was grown in nude mice, and tumor samples were collected either before ( $t = 0$ ) or 1, 2, or 4 weeks after castration. Total RNA was isolated and then used in Northern analysis with the same probes. Note that CWR22 week 2 lane was underloaded (compare 18 S RNA). In parallel, four independent lines of the androgen-independent human prostate cancer xenograft CWR22R were grown in nude mice, tumors were collected, and total RNA was isolated and used in Northern analysis with STAMP1 or the androgen target gene NKX3.1 cDNAs as probes, as indicated.

four independent, relapsed derivatives of CWR22 tumors, named CWR22R (18), which are representative of advanced prostate cancer. Interestingly, there was no STAMP1 expression in the androgen-independent prostate cancer cell lines PC-3 or DU145, similar to that observed for NKX3.1 (Fig. 2B). In contrast, there was significant STAMP1 expression in tumors from all four independent CWR22R xenograft lines tested, ranging between ~30 and 60% of that observed in LNCaP cells. A similar overexpression pattern was also observed for NKX3.1 (Fig. 2B), consistent with previous findings (25). These data suggest that STAMP1 expression may be deregulated once prostate cancer progresses from an androgen-dependent to an androgen-independent state.

**Intracellular Localization of STAMP1**—To gain insight into the cellular localization of STAMP1, we labeled it with GFP to generate GFP-STAMP1. Such use of GFP fusion proteins has recently become a standard method to assess intracellular localization and dynamics of proteins (26, 27). COS-1 cells were transiently transfected with GFP-STAMP1, fixed, and processed for confocal microscopy. The series of 11 confocal sec-

tions along the z-axis were collected through a single cell at nominal 100-nm intervals. Three of the consecutive sections and projection of all 11 sections are shown in Fig. 3A. In all 11 z-plane sections, GFP-STAMP1 showed bright juxtanuclear distribution patterns, characteristic of the Golgi complex. Additionally, GFP-STAMP1 was dispersed in dots with variable size throughout the cytoplasm and at the cell periphery (Fig. 3, z-7, projection). Some of these bright fluorescent spots were tubular (z-6, arrow) or vesicular (z-5, arrow) in morphology.

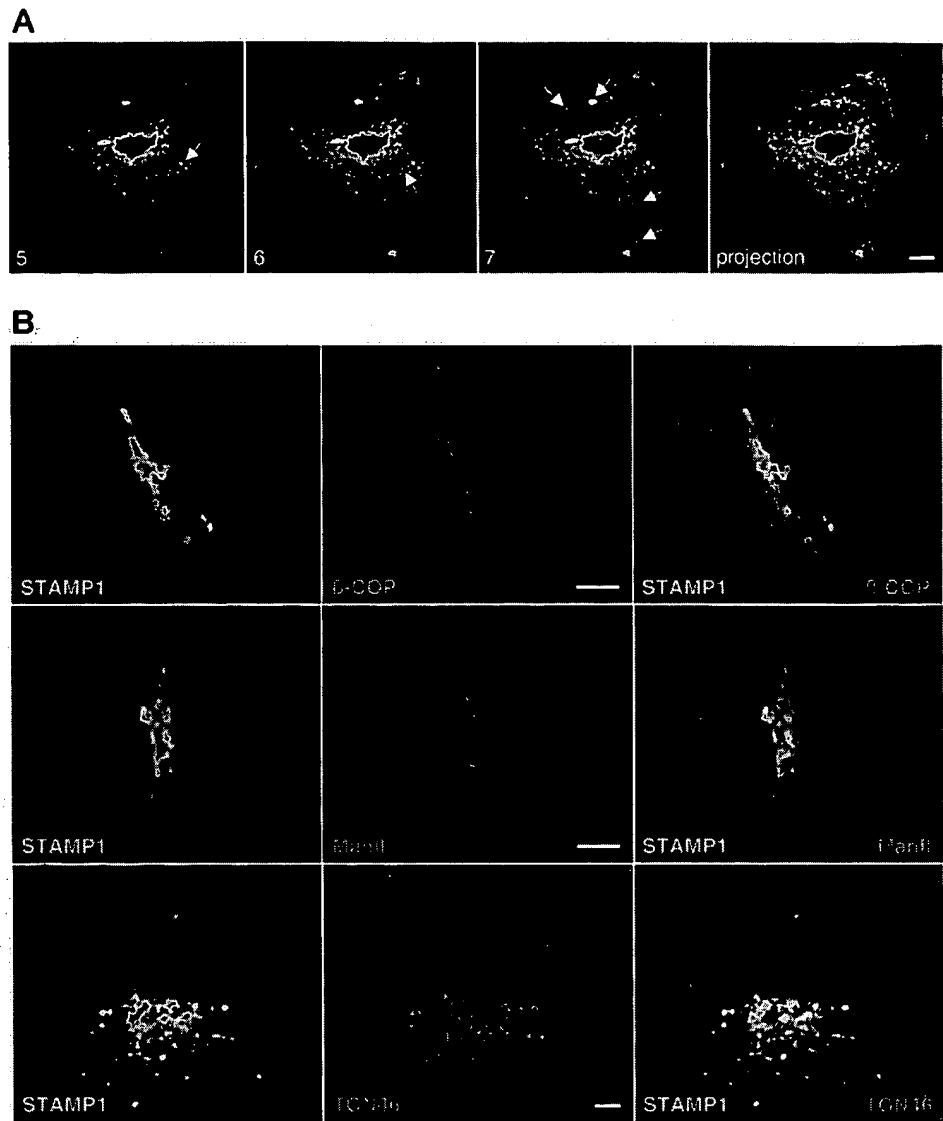
To determine more directly whether GFP-STAMP1 was localized to the Golgi complex, we compared its intracellular distribution with those of two well characterized Golgi markers, the medial Golgi enzyme mannosidase II (ManII) (28) and the coat protein  $\beta$ -COP (29). GFP-STAMP1 was transfected into COS-1 cells, which were then fixed and labeled with the appropriate primary and secondary antibodies, and then optical sections were analyzed by laser scanning confocal microscopy. As shown in Fig. 3B, the distribution of GFP-STAMP1 extended throughout the Golgi complex, as evidenced by significant colocalizations with both ManII and  $\beta$ -COP. However, some areas of non-overlap between the GFP-STAMP1 and both Golgi markers were observed, suggesting that STAMP1, at least in part, is differentially localized within the Golgi complex compared with these two markers. Virtually identical results were obtained by visualization of endogenous STAMP1 in LNCaP cells using a STAMP1-specific antiserum (data not shown).

Because GFP-STAMP1 appeared to be associated with vesicular tubular structures (VTS) (Figs. 3A and 4), we assessed whether it may be more specifically localized to the TGN, an important site for the sorting of proteins destined to the plasma membrane, secretory vesicles, or lysosomes (30–32). To that end, we used an antibody against TGN46, a TGN resident protein that shuttles between the TGN and the plasma membrane, (33, 34) in immunofluorescence microscopy experiments as above. As shown in Fig. 3B, GFP-STAMP1 extensively (~80%) co-localized with TGN46, greater than that observed with ManII and  $\beta$ -COP, suggesting that in the Golgi complex STAMP1 is primarily localized to the TGN.

**STAMP1 Shuttles Between the Golgi and the Plasma Membrane and Colocalizes to the Endosomes**—To gain insight into the possible function of STAMP1, the kinetic properties of GFP-STAMP1 distribution and trafficking were studied using confocal time-lapse imaging in living cells. COS-1 cells were transfected with GFP-STAMP1. 18 h after transfection, images were captured from live cells every 20 s at 37 °C by confocal laser scanning microscopy (see QuickTime movie sequence at [www.biologi.uio.no/mcb/fs/project2.html](http://www.biologi.uio.no/mcb/fs/project2.html)).

As shown in Fig. 4, there was anterograde-retrograde trafficking of GFP-STAMP1 to and from the Golgi complex predominantly in the form of VTS. Some of the VTS followed straight or curvilinear paths, some moved in a stop-and-go fashion, and some showed saltatory movements (see Quicktime movie). The mobile structures indicated at the top panel (white arrows) extended away from and then retracted back to the Golgi. The VTS in the middle panel and the first image in the lower panel (red arrows) detached from the Golgi complex, paused, and moved toward the cell periphery until it disappeared at the cell edge, suggesting that STAMP1 is associated with the secretory pathway. The VTS (yellow arrow) in the lower panel moved from the cell periphery toward the Golgi body, suggesting that STAMP1 is also associated with the endocytic pathway.

To probe whether GFP-STAMP1 was associated with the endocytic pathway, we compared the intracellular distribution of GFP-STAMP1 with that of the early endosome protein EEA1 (35). GFP-STAMP1 was transfected into COS-1 cells that were then fixed, immunostained with EEA1 antibodies, and ob-



**FIG. 3. Intracellular localization of GFP-STAMP1.** A, COS-1 cells were transiently transfected with GFP-STAMP1. Cells were fixed and prepared for confocal microscopy as described under "Materials and Methods." The series of 11 confocal sections were collected through a single cell along the z-axis at 100-nm intervals, three of which are shown along with the projection of all (*projection*). Arrows indicate VTS in different sizes, shapes, and locations. Bar, 5  $\mu$ m. B, colocalization of GFP-STAMP1 with Golgi markers. GFP-STAMP1 was transfected into COS-1 cells that were fixed and labeled with antibodies against well characterized Golgi and TGN markers:  $\beta$ -coat protein ( $\beta$ -COP), ManII, and TGN46. Green GFP-STAMP1 fluorescence and red (Texas Red-labeled secondary antisera)  $\beta$ -COP, ManII, and TGN46 fluorescence were detected by confocal laser microscopy. The right panel shows the overlay images with yellow/orange staining indicating the regions of overlap. Bars, 5  $\mu$ m. Note that the images with TGN46 were obtained with lower objective power.

served by laser scanning confocal microscopy. As shown in Fig. 5, EEA1 had similar intracellular distribution in both transfected and untransfected cells. Furthermore, GFP-STAMP1 significantly colocalized with EEA1 both in the cell periphery and also in the perinuclear area (Fig. 5, indicated by arrows), suggesting that STAMP1 is associated with early endosomes and the endocytic pathway.

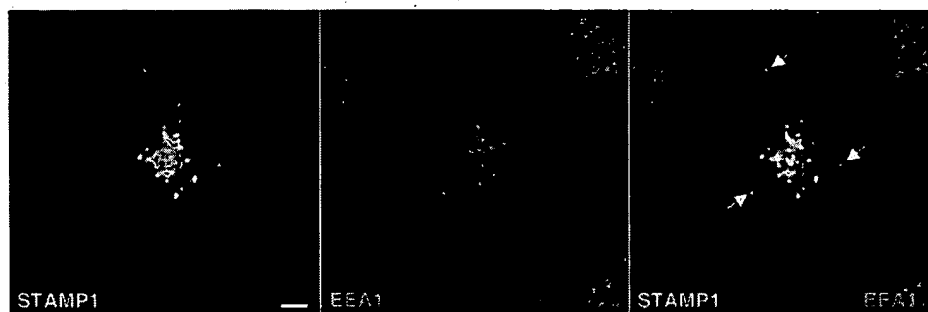
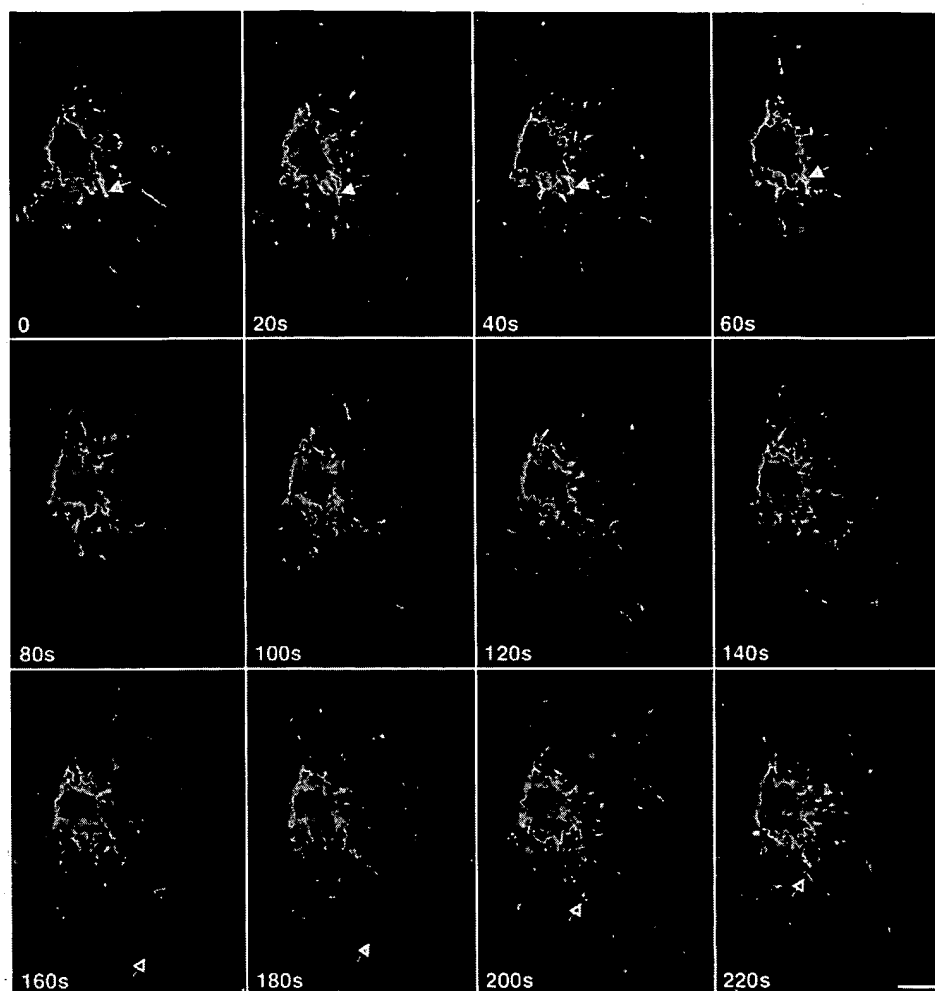
**Analysis of STAMP1 Expression in Normal Versus Cancerous Prostate**—Because STAMP1 is highly enriched in the prostate compared with other tissues, we studied its expression in normal prostate and compared it with prostate cancer. To assess the location of STAMP1 expression in prostate tissue and the possible differences in normal compared with neoplastic cells, we performed *in situ* hybridization analysis on six embedded tissue blocks from prostatectomy specimens (data not shown) or a tissue microarray that contained 46 tissue samples from 13 prostatectomy cases (example shown in Fig. 6). STAMP1 was expressed exclusively in the epithelial cells of the prostate, both in normal and tumor glands (Fig. 6A). However, the level of STAMP1 expression was higher in the tumor glands, ~2.5-fold overall, compared with normal epithelium (Fig. 6B). These data indicate that STAMP1 may directly contribute to, or be a marker for, the progression of prostate cancer.

## DISCUSSION

Proteins that contain six transmembrane domains have key roles in a variety of important physiological processes. For example, they function as ion channels (36), as signal transducers of painful stimuli (37), as water channels or aquaporins (38), as lipid phosphate phosphatases or LPPs (39), and as ATP-binding cassette (ABC) transporters and multidrug resistance (MDR) proteins (40). Given these important functions, it is not surprising that deregulation/mutation of some of these proteins is implicated in the pathogenesis of major human diseases such as Alzheimer's (41) and Tangier disease (42). Because STAMP1 is highly prostate-specific for expression, it is tempting to speculate that deregulation or mutation of STAMP1 may have a role in the pathogenesis of prostate cancer.

STAMP1 shows significant similarity to three recently identified genes. Although there is high sequence similarity with both TIARP and pHyde over the whole ORF, the similarity with STEAP is restricted to the predicted transmembrane domain in the C terminus. In fact, STEAP is a smaller protein and does not have an N-terminal region compared with STAMP1 and TIARP. Both pHyde and STEAP have previously been implicated in the prostate; pHyde for its ability to induce growth arrest and apoptosis of prostate cancer cell lines (21)

**FIG. 4. Time-lapse analysis of GFP-STAMP1 trafficking in living cells.** A COS-1 cell that transiently expresses GFP-STAMP1 was observed by live-cell confocal microscopy at 37 °C. Twelve consecutive images were taken at 20-s intervals. The *upper panel* shows a tubular structure extending out and retracting back to the Golgi body (*white arrows*). In the *middle panel* and the first image in the *lower panel* (160 s), *red arrows* indicate the translocation of a tubular particle away from the Golgi body to the cell periphery. In the *lower panel*, *yellow arrows* point to the movement of a tubular particle from the edge of the cell toward the Golgi body. Note that the results shown are representative of multiple time-lapse analyses, and the changes in the images are not due to movement from the plane of focus. Bar, 5  $\mu$ m.



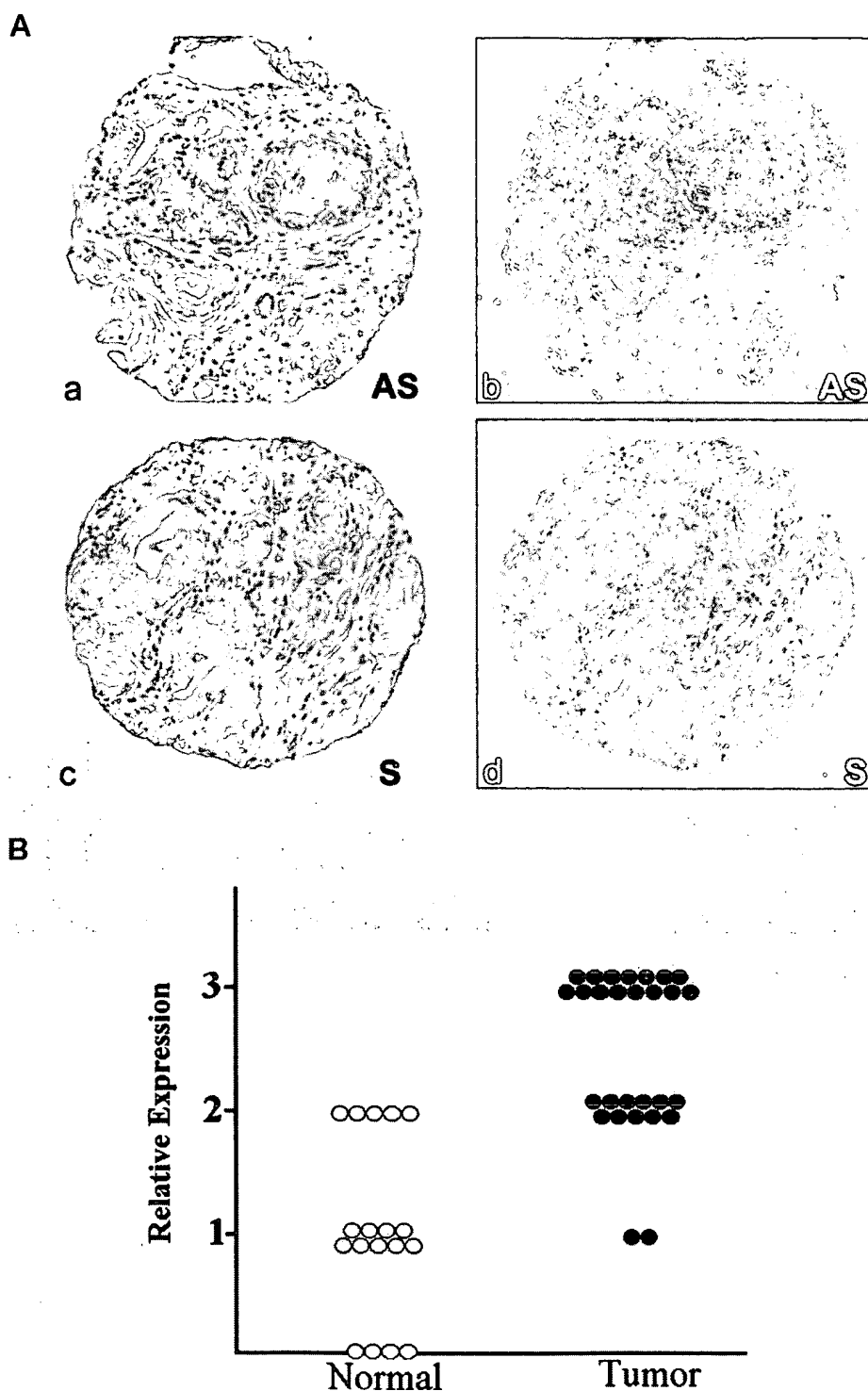
**FIG. 5. GFP-STAMP1 colocalizes to early endosomes.** Colocalization of GFP-STAMP1 with the early endosomal marker EEA1 as detected by immunofluorescence microscopy. GFP-STAMP1 was transfected into COS-1 cells that were fixed and labeled with antibodies against the well characterized early endosomal marker EEA1. Green GFP-STAMP1 fluorescence and red (Texas Red-labeled secondary antiserum) EEA1 fluorescence were detected by confocal laser microscopy. The *right panel* shows the overlay images with *yellow* staining indicating the regions of overlap. Arrows indicate examples of the vesicular structures in the cell periphery, which contain both EEA1 and STAMP1. Bar, 5  $\mu$ m.

and STEAP for its significantly prostate-enriched expression pattern (23). There is no information on the expression of TIARP in the prostate to date, but our preliminary analysis with the human TIARP cDNA suggests that it has a ubiquitous expression profile, similar to that for human pHyde.<sup>3</sup> Based on the secretory/endocytic pathway localization of GFP-STAMP1, TIARP, pHyde, and STEAP may also be involved in intracellular trafficking pathways. However, there are some differences in their subcellular distribution patterns compared with STAMP1, suggesting that they have distinct functions in the

cell. Further work is in progress to assess whether the sequence similarities of these proteins also extend to an overlap in cellular localization and function.

To our knowledge, STAMP1 is the first six transmembrane protein described to date that is localized to the Golgi and TGN. Most Golgi-resident proteins studied so far are transmembrane proteins that share a common structure, the majority of which have a single type II transmembrane domain (43, 44). Expression of STAMP1 as a six transmembrane protein in the Golgi suggests that it may have unique functions compared with the other Golgi-resident proteins identified so far. Photobleaching experiments provided evidence that GFP-STAMP1 is rapidly exchanged between different parts of the Golgi with kinetics

<sup>3</sup> K. Korkmaz, C. G. Korkmaz, and F. Saatcioglu, unpublished results.



**FIG. 6. *In situ* analysis of STAMP1 expression in normal versus neoplastic prostate tissues.** *In situ* hybridization of paraffin-embedded human prostate cancer specimens (data not shown) or tissue arrays derived from them were performed using probes derived from either sense or antisense strands of STAMP1. Hematoxylin and eosin staining is shown to the left and *in situ* images are to the right. The other panels are photographed using dark-field microscopy after a 14-day exposure. Positive hybridization is seen as bright spots. **A**, *in situ* analysis on prostate cancer tissue array. **S**, sense probe; **AS**, antisense probe. **B**, comparison of STAMP1 expression in normal versus tumor cells in the prostate by *in situ* analysis. Sections on a prostate array were scored by a pathologist and assigned a relative value of 0, 1, 2, or 3 according to increasing levels of STAMP1 expression in 18 normal and 28 tumor sections. Each score is presented: normal, open circles; tumor, filled circles. Student's *t* test analysis indicates that the means of the normal and tumor samples are significantly different ( $p < 0.001$ ).

consistent for it being a transmembrane protein (data not shown), supporting the notion that it indeed functions as a transmembrane protein in the Golgi. In addition, in the presence of Brefeldin A, a known inhibitor of the formation of TGN (45, 46), GFP-STAMP1 localization to TGN is inhibited which is reversible (data not shown). Further work is needed for the biochemical and functional characterization of STAMP1 in the Golgi and TGN.

The Golgi complex has a central role in the secretory pathway (30, 31, 47, 48). It is involved in the processing and sorting of proteins and lipids to their final destinations, *i.e.* to the cell surface, secretory granules, endosomes, or recycling back to the

endoplasmic reticulum. Because prostate is a major secretory organ (49), trafficking through the Golgi is expected to be important and tightly regulated in prostate cells. However, in contrast to some other specialized organs, such as the pancreas (50), there is no specific knowledge about functioning of the Golgi in the prostate. For example, it is possible that the secreted molecules of the prostate, such as PSA, are modified or sorted in a regulated fashion in the Golgi complex. Resident enzymes of the Golgi in the prostate, such as STAMP1, may therefore have an important role in this type of regulation. Alternatively, STAMP1 may be a receptor for an endogenous or exogenous ligand that may be delivered to different intracellu-



lar compartments through the secretory and endocytic pathways to be involved in cellular processes. Further studies are required to assess these possibilities.

An interesting property of *STAMP1* expression profile is that it is not expressed in the AR-negative prostate cancer cell lines PC-3 and DU145 but is expressed at high levels in the AR-positive cell line LNCaP. It is also present in the CWR22 and CWR22R xenografts, which are AR-positive. Thus, expression of *STAMP1* is correlated with the presence of a functional AR in the cell. Whether AR is directly involved in *STAMP1* expression will require the characterization of *STAMP1* flanking sequences and further functional studies. Another related area to pursue is the expression of *STAMP1* and its variants in the different cell populations that are found in the prostate. Laser capture microdissection coupled to quantitative RT-PCR is in progress to look for these possible differences.

It has been known for over 60 years that androgens play a key role both in the development and maintenance of the normal prostate and the initiation and progression of prostate cancer (3). This is the basis for androgen withdrawal therapy, which unfortunately fails in most cases after a few months or years. At this point, there is no effective therapy and prognosis for survival is extremely poor (3–5). Because *STAMP1* expression may be increased during the progression to this advanced state, it may prove to be a useful tool in diagnostic and therapeutic applications for prostate cancer, in addition to helping define the molecular dynamics of the prostate cancer cell.

**Acknowledgments**—We thank Drs. J. Lippincott-Schwartz, J. S. Bonifacino, and T. Misteli for antisera, Dr. T. Pretlow and J. Giaconia for CWR22 and CWR22R tumor samples, C. Tam for technical assistance, and Dr. T. Misteli for advice, helpful discussions, and critically reading the manuscript.

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## Dissociation Between Androgen Responsiveness for Malignant Growth vs. Expression of Prostate Specific Differentiation Markers PSA, hK2, and PSMA in Human Prostate Cancer Models

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**BACKGROUND.** A detailed understanding is evolving as to how androgen receptor (AR) functions as a transcriptional regulator via its binding to androgen response elements (ARE) within promoter and enhancer regions of prostate-specific differentiation markers such as PSA, hK2, and PSMA. It has been assumed that an understanding of regulation of expression of these marker proteins would also provide an understanding of the mechanisms whereby AR interactions regulate proliferation and survival of malignant prostate cells. In order to validate this hypothesis, we used a series of human prostate cancer models [i.e., LAPC-4, CWR22Rv1, MDA PCA-2b, LNCaP, and C4-2B (derived from LNCaP)] to test whether there is a consistent concordance between androgen responsive regulation for malignant growth vs. regulation of expression of prostate differentiation specific markers PSA, hK2, and PSMA.

**METHODS.** In order to define androgen growth responsiveness in vivo, human prostate cancer cell lines were inoculated as xenografts into intact vs. surgically castrated adult male nude mice and the subsequent tumor growth response monitored. To assess androgen regulation of PSA and hK2 expression in these cell lines, the concentration of PSA and hK2 in the conditioned standard media and charcoal stripped media  $\pm$  androgen from each cell line was determined using an immunoassay system. PSMA enzymatic activity was determined using the PSMA substrate <sup>3</sup>H N-acetylaspartylglutamate (<sup>3</sup>H NAAG).

**RESULTS.** Wild-type AR expressing LAPC-4 cells are androgen responsive for their in vivo growth. This cell line is also androgen sensitive for the expression of both PSA and hK2 in vitro and express PSMA. CWR22Rv1 cells have a mutated AR and are androgen responsive for growth in vivo and androgen sensitive for hK2 but not PSA expression. CWR22Rv1 produce ~1.4-fold more PSA, ~18-fold more hK2, and have 21-fold higher PSMA activity than LAPC-4 cells. MDA PCA-2b cells are androgen responsive for growth in vivo and androgen sensitive for PSA expression. MDA PCA-2b cells produce ~250-fold more PSA but almost equivalent amounts of hK2 compared to LAPC-4 and have ~19-fold higher PSMA activity. Both late passage LNCaP and C4-2B are androgen independent for growth in vivo but remain androgen sensitive for both PSA and hK2 expression. LNCaP cells produce ~50-fold more PSA, ~35-fold

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more hK2, and have 28-fold higher PSMA activity compared to LAPC-4. C4-2B cells produce ~80-fold higher levels of PSA, ~250-fold higher levels of hK2. C4-2B also the highest PSMA activity of the cell lines with 105-fold higher PSMA activity than LAPC-4 and ~4-fold higher activity than late passage LNCaP cells.

**CONCLUSIONS.** Androgen can coordinately regulate both the tumor growth and expression of prostate specific marker genes as observed for the LAPC-4 human prostate cancer cells. Such coordinated regulation, however, is not universal. In all of the other cell lines, there is a dissociation between androgen responsive regulation of malignant growth vs. regulation of expression of prostate specific markers PSA and hK2. In addition, PSMA activity in these cell lines increases as cells become more androgen independent for growth *in vivo*. These results emphasize that tumor growth and the expression of the specific secretory genes are independently regulated molecular events even if they share a requirement for androgen and/or AR function. Additional independent mechanisms occur in prostate cancer cells for regulation of expression for even the highly related *PSA* and *hK2* genes. Further studies are needed to clarify the mechanisms for androgen ligand-independent, AR-dependent regulation of the genes that directly effect the growth of androgen (i.e., ligand) independent prostate cancer cells. Unfortunately, the data in this present report do not validate the use of the *PSA* or *hK2* gene as surrogates for a model system for such critically important mechanistic studies.

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**KEY WORDS:** prostate cancer; prostate-specific antigen (PSA); human glandular kallikrein 2 (hK2); prostate specific membrane antigen (PSMA); androgen

## INTRODUCTION

Androgen is the major growth factor for the prostate epithelium. This is because it stimulates the rate of epithelial proliferation while inhibiting the rate of epithelial cell death [1]. Normally these two processes are balanced so that the epithelium is in a state of self-renewal in which neither regression nor overgrowth of the prostate epithelium occurs [1]. Androgens regulate this balance through the occupation of the androgen receptor (AR) by its cognate ligand within the prostate stromal cells. Such occupancy stimulates the transcription of specific genes resulting in the secretion of soluble peptide paracrine factors by the stromal cells [2–4]. Once secreted, these paracrine factors cross the basement membrane of glandular acini where they bind to epithelial cell receptors initiating the regulation of proliferation and survival of the latter cells [2–4]. While not regulating the proliferation/survival of prostate epithelial cell directly, androgen occupation of the AR within the nuclei of these cells does regulate expression of prostate-specific marker proteins such as Prostate-Specific Antigen (PSA), Human Glandular Kallikrein 2 (hK2), and Prostate-Specific Membrane Antigen (PSMA) [5–8]. During the malignant progression of these prostate epithelial cells, molecular changes occur that enable ligand/AR interactions within the malignant epithelial cells to regulate directly the expression of prostate marker proteins such as PSA, hK2, and PSMA, in addition to the autocrine production of growth and survival factors without stromal requirement [9].

This conversion from a paracrine to an autocrine mechanism of androgen action during prostate carcinogenesis is consistent with why prostate cancer cells are so responsive initially to androgen ablation therapy, even when in distant metastatic sites. It likewise provides a conceptual framework for how androgen ablation failure develops. Such failure could occur due to genetic alteration in the AR itself and/or its co-activators to effect transcription of critical growth/survival genes [10–16]. To study this possibility, the mechanism for how AR regulates the expression of *PSA* and *hK2* have been used as model genes. Over the last several years a detailed understanding is evolving as to how AR functions as a transcriptional regulator via its binding to androgen response elements (ARE) within promoter and enhancer regions of prostate marker proteins such as *PSA*, *hK2*, and *PSMA* [5–8,17–24]. It has been assumed that an understanding of regulation of expression of these marker proteins would also provide an understanding of the mechanisms whereby AR interaction regulates malignant proliferation and survival. While the assumption that the mechanisms underlying regulation of marker protein expression may be similar to those underlying regulation of malignant cell proliferation and survival is logical, it has not been validated experimentally.

In order to validate this hypothesis, we used a series of human prostate cancer models [i.e., LAPC-4, CWR22Rv1, MDA PCA-2b, LNCaP, C4-2B (derived from LNCaP)] to test whether there is a consistent concordance between androgen responsive regulation of malignant growth vs. regulation of expression of

prostate differentiation specific markers PSA, hK2, and PSMA. In the process, we have also characterized the expression of PSA, hK2, and PSMA by this panel of human prostate cancer cell lines in order to provide a reference for investigators who are using these lines to develop targeted therapies for prostate cancer or who are studying the role of these proteases in prostate cancer tumor biology.

## MATERIALS AND METHODS

### Materials

The synthetic androgen R1881 was from Amersham. All other reagents and chemicals, unless otherwise specified, were from Sigma Chemical (St. Louis, MO).

### Cell Lines

The previously characterized LNCaP human prostate cancer line [25] was obtained from ATCC (Rockville, MD); CWR22Rv1 was provided by Dr. Jacobberger (Case Western Reserve University, Cleveland, OH) [26]; C4-2B was from UroCor [27]. These lines were maintained by serial passage in RPMI 1640 media (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Bio-Whittaker, Walkersville, MD). MDA PCa-2b human prostate cancer cells were provided by Dr. Nora Navone (MD Anderson Cancer Center, Houston, TX) and maintained in HPC-1 media (AthenaES, Baltimore, MD) containing 20% FBS [28]. The HPC-1 media contains 280 nM hydrocortisone and 0.1 nM DHT. LAPC-4 human prostate cancer cells were provided by Dr. Charles Sawyers (UCLA, Los Angeles, CA) and were maintained in Iscove's media (Gibco) containing 10% FBS and 1 nM R1881 [29]. All standard media included 100 U/ml penicillin G, and 100 U/ml streptomycin sulfate and all cells grown in 5% CO<sub>2</sub>/95% air at 37°C. To assess marker protein expression sensitivity to androgen, cell lines were grown in respective phenol red free standard medias (i.e., RPMI or Iscove's) supplemented with 10% dextran, charcoal stripped serum (Gibco)  $\pm$  1 nM R1881.

### In Vivo Growth Assays

Cell lines were grown in standard media, pelleted, and then resuspended in undiluted Matrigel (Collaborative Biomedical, Bedford, MA) to a concentration of  $2 \times 10^7$  cells/ml. Two hundred  $\mu$ l of the tumor cell suspensions were inoculated subcutaneously into the flank of intact or castrated male nude mice. Once palpable, tumors were measured at indicated times using vernier calipers and tumor volume calculated using the formula length  $\times$  width  $\times$  height  $\times$  0.5236. At the end of the experiments, animals were sacrificed by CO<sub>2</sub> overdose. All animal studies were performed

according to protocols approved by the Johns Hopkins Animal Care and Use Committee.

### PSA and hK2 Measurements

Total PSA and total human kallikrein 2 (thK2) were analyzed using Hybritech assays on the Beckman Access Immunoassay System (Beckman Coulter, Inc., Brea, CA). The thK2 assay is a two-site immunoenzymatic utilizing dual monoclonal antibodies and a recombinant wild-type hK2 calibrator that have been described previously [30]. The assay measures both free hK2 and hK2 complexes and has minimal cross-reactivity with PSA. The assay is for research use only.

### Western Blot

To perform western blots a PSMA fusion protein (Fc-PSMA) was used as a positive control. The production of the Fc-PSMA vector and characterization of the Fc-PSMA fusion protein have been described previously by Lo et al. [31]. Fc-PSMA produced and purified in this manner was provided by Lexigen Pharmaceuticals (Lexington, MA) [31]. A 12% SDS gel was used for separation and then western blot analysis performed as previously described. Primary antibody was a Y-PSMA, monoclonal antibody available from Yes Biotech (Mississauga, Ontario) at 1:1000 dilution.

### PSMA Enzymatic Assays

The enzymatic activity assay for PSMA was adapted as previously described by Tiffany et al. [32]. Lysates were added to PSMA assay buffer (10 mM CoCl<sub>2</sub>, 50 mM Tris, pH 7.4). Following a 10 min incubation at room temperature, *N*-acetyl-aspartyl-<sup>3</sup>H glutamate (<sup>3</sup>H-NAAG) (NEN, Boston, MA) was added to final concentration of 0.025  $\mu$ M and reactions incubated for various times at room temperature. The concentration of the reaction product <sup>3</sup>H-Glu was isolated determined by passing an aliquot of the reaction mixture through an AG 1-X8 ion exchange resin (Bio-Rad). A standard curve was plotted using increasing concentration of <sup>3</sup>H-NAAG in order to convert measured counts to pmoles <sup>3</sup>H-glutamate released.

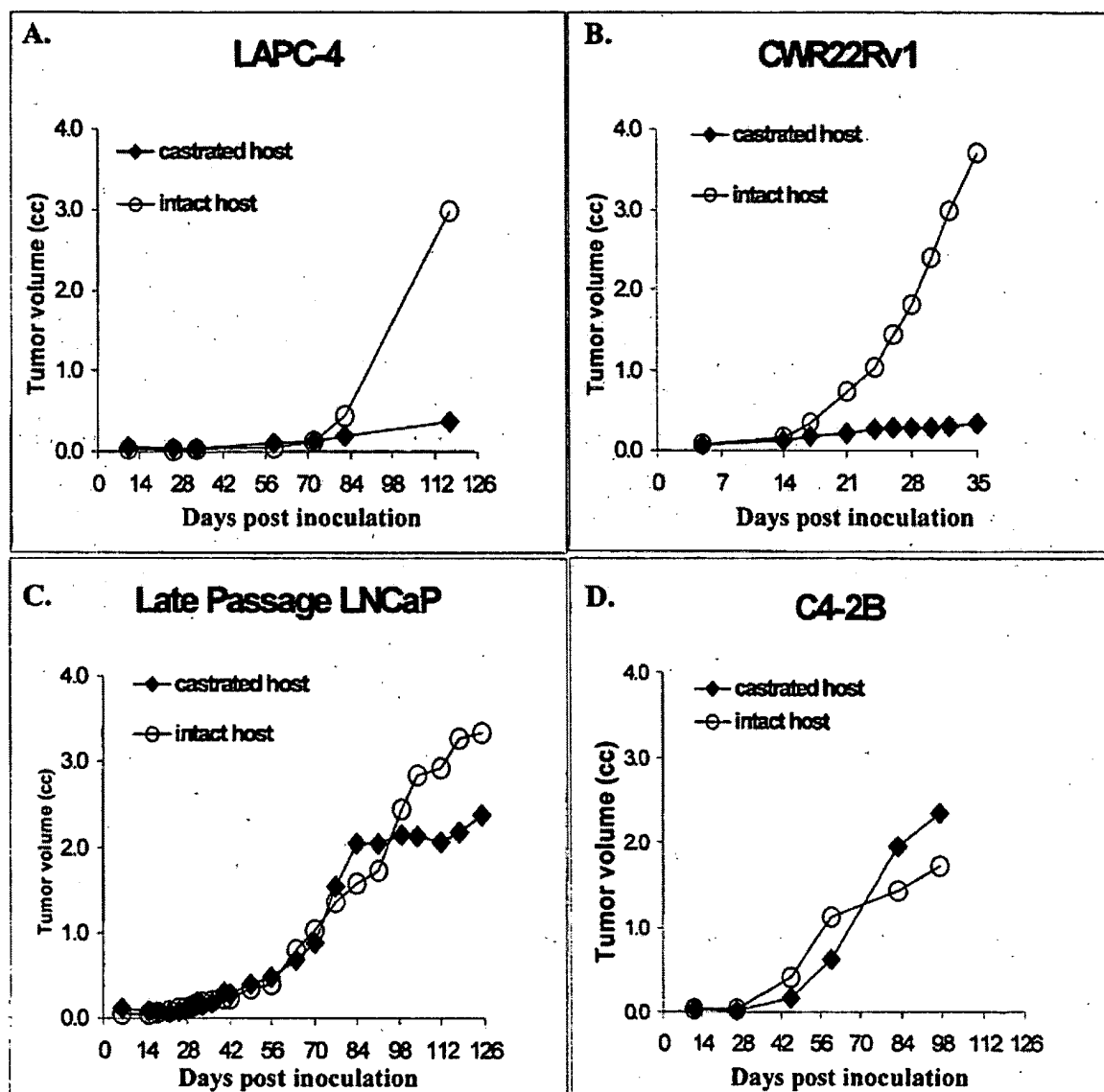
## RESULTS

### Relationship Between In Vivo Growth Response and PSA and hK2 Expression

The most fundamental characteristic of a lethal prostate cancer is that its rate of malignant cell proliferation exceeds its rate of cell death [33]. This imbalance results in the continuous net growth of the cancer. Thus, to develop effective therapies for prostate cancer, in vitro model systems are critical since they

allow for both mechanistic studies for therapeutic target discovery and rapid throughput screening to identify compounds for drug development. In order to be relevant, however, such *in vitro* systems must reflect the critical malignant *in vivo* biology. For prostate cancer models, this means defining growth response to androgen in the most clinically appropriate setting, i.e., *in vivo*. Since androgen ablation is the "gold standard" for defining "androgen responsiveness" of clinical cancers, human prostate cancer cell lines were inoculated as xenografts into intact vs. surgically castrated adult male nude mice and the subsequent tumor growth response monitored.

The LAPC-4 human prostate cancer cell line was the first line evaluated for *in vivo* growth response and PSA and hK2 expression in the presence and absence of androgen. The LAPC-4 cell line was derived by direct transfer of surgical samples from patients into immune-deficient male SCID mice [29]. These cells express wild-type AR [29]. When inoculated into nude mice, late passage LAPC-4 cells produce xenografts that grow in castrate hosts, although at a markedly slower rate than in intact animals, Figure 1A. Thus these cells are androgen responsive for growth. Although LAPC-4 cells produce low basal levels of PSA and hK2, the addition of androgens still results



**Fig. 1.** Growth of human prostate cancer cells in intact and castrated male nude mice. **A:** LAPC-4; **(B)** CWR22Rv1; **(C)** LNCaP; **(D)** C4-2B. Each cell line mixed with undiluted Matrigel and  $2 \times 10^6$  cells (200  $\mu$ l volume) injected subcutaneously into flank of intact or previously castrated nude mice. Tumor volume measured at indicated times using vernier calipers and volume calculated using formula length  $\times$  width  $\times$  height  $\times$  0.5236.

in significant increase in both PSA and hK2 production, Table I. Therefore, wild-type AR expressing LAPC-4 cells are androgen growth responsive and sensitive to androgen for marker protein expression, Table I. Thus, for this cell line there is a concordance between androgen responsive regulation of malignant growth vs. regulation of expression of prostate specific markers PSA and hK2.

Recently, Navone et al. [28] established two cell lines (MDA PCa-2a and MDA PCa-2b) from a patient who had disease progression following androgen ablation. These cells express PSA, AR, and grow in vitro. Cells from these lines produced tumors in nude mice when injected either s.c. or orthotopically (intraprostatic) [28]. The MDA PCA-2b cell line expresses abundant AR that has two mutations in the ligand binding domain of the AR gene [14]. Even with these AR mutations, established MDA PCA-2b cancers growing in intact male nude mice undergo regression following castration, with ~40% of tumors eventually relapsing [11,14,34]. Thus, the in vivo growth of MDA PCA-2b is androgen responsive. In vitro, MDA PCA-2b cells express high amounts of PSA (i.e., ~250-fold higher than LAPC-4) but, equivalent amounts of hK2 when grown in standard media, Table I. Krishnan et al. [35] have previously demonstrated that the PSA expression by MDA PCA-2b cells is androgen responsive in vitro. On the basis of these results the MDA PCA 2b cells are androgen responsive for growth and androgen

sensitive for PSA expression, Table I. Thus, the concordance between androgen responsiveness for growth and androgen sensitivity for marker expression does not require wild-type AR expression.

The next cell line to be evaluated was the CWR22Rv1 human prostate cancer cell line, derived from a xenograft that was serially propagated in mice after castration-induced regression and relapse of the parental, androgen-dependent CWR22 xenograft [26]. CWR22Rv1 cells have a mutated AR [36]. Early passage CWR22Rv1 xenografts grow markedly more rapidly in intact nude mice than the LAPC-4 cells, Figure 1B. In contrast, however, CWR22Rv1 xenografts grow much slower in castrated vs. intact hosts, Figure 1C demonstrating that their in vivo growth is responsive to androgen. CWR22Rv1 produce ~1.4-fold more PSA and ~18-fold more hK2 than LAPC-4 cells when grown in standard media, Table I. CWR22Rv1 cells also produce low basal levels of PSA and hK2 in androgen depleted media, Table I. Addition of androgen to this depleted media resulted in no change (i.e., <2-fold) in PSA but a 2.8-fold increase in hK2 production, Table I. The CWR22Rv1 cells, therefore, are androgen responsive for growth and androgen sensitive for hK2 but not PSA expression, Table I.

Finally, to assess relationship between androgen responsiveness for growth and androgen sensitivity of prostate marker protein expression in "androgen independent" models we used late in vitro passage

**TABLE I. Androgen Growth Responsiveness In Vivo and Regulation of Expression of PSA and hK2 in Human Prostate Cancer Cell Lines**

Cell line <sup>a</sup>	Passage number	AR status	In vivo response to androgen ablation	Media	PSA (ng/10 <sup>7</sup> cells/day) <sup>d,e</sup>	hK2 (ng/10 <sup>7</sup> cells/day) <sup>d,e</sup>
LAPC-4	130	Wild type	Responsive	Standard <sup>a</sup>	8.5 ± 1	0.6 ± 0.2
				R1881- <sup>b</sup>	8.5	0.2
				R1881+ <sup>b</sup>	29.8	1.5
MDA PCA-2b	41	Mutant	Responsive	Standard <sup>a</sup>	2155 ± 355	0.5 ± 0.2
CWR22Rv1	80	Mutant	Responsive	Standard <sup>a</sup>	12 ± 3	11 ± 2
				R1881- <sup>b</sup>	13	4
				R1881+ <sup>b</sup>	19	11
LNCaP	150	Mutant	Independent	Standard <sup>a</sup>	427 ± 37	22 ± 6
				DHT+ <sup>c</sup>	2314 ± 620	64 ± 41
C4-2B	52	Mutant	Independent	Standard <sup>a</sup>	702 ± 30	149 ± 30
				R1881- <sup>b</sup>	855	94
				R1881+ <sup>b</sup>	2158	238

<sup>a</sup>Standard media for LNCaP, C4-2B, and CWR22Rv1 cells is RPMI + 10% FBS; MDA PCA-2b is HPC-1 + 20% FBS; LAPC-4 is grown in Iscove's + 10% FBS + 1 nM R1881.

<sup>b</sup>10% Dextran, charcoal stripped, phenol red free, 10% FBS media ± R1881 (1nM).

<sup>c</sup>DHT (100 nM) added to standard media.

<sup>d</sup>PSA and hK2 levels measured from media conditioned for 4 days.

<sup>e</sup>ng/10<sup>7</sup>/day cells determined by [(ng/ml × total mls media)/cell number × 10<sup>7</sup>]/no. of days.

(i.e., >150) LNCaP cells and the LNCaP subline C4-2B. The use of late passage LNCaP cells was based upon the demonstration by Lin et al that serial passage (i.e., >80) of LNCaP in standard tissue culture media with no manipulation of hormonal levels selects for androgen independent cancer cells as evidenced by their ability to grow equally well in either intact or castrated animals [37]. We have confirmed this observation using late passage (i.e., >150) LNCaP cells. Inoculation of such late passage cells produces cancers that grow identically in intact vs. castrated mice, Figure 1C. In fact, the growth of these serially passaged (i.e., unmanipulated) LNCaP cells in intact and castrate animals is identical to growth of androgen independent C4-2B cells (Fig. 1D) an LNCaP subline developed through a complicated series of manipulations involving multiple rounds of alternating in vitro and in vivo passage in an androgen ablated environment [27]. Like LNCaP, C4-2B cells must also be co-inoculated with Matrigel for xenografts to grow reproducibly in nude mice. In addition, like LNCaP, the C4-2B cells have a mutated but functional AR [10,27]. These results document that androgen growth independent LNCaP cells are selected simply by serial passage without any requirement for the complicated androgen manipulation required to produce the C4-2B subline. As summarized in Table I, the late passage LNCaP cell line produces ~50-fold more PSA and ~35-fold more hK2 than LAPC-4. The C4-2B cell lines produce ~80-fold higher levels of PSA and ~250-fold higher levels of hK2 in standard media than LAPC-4 cells. C4-2B cells also have high basal levels of PSA and hK2 even when cultured in charcoal stripped, androgen depleted media, Table I. Both late passage LNCaP and C4-2B cells upregulate PSA and hK2 expression in the presence of androgen, Table I. Late passage LNCaP and C4-2B cell lines are therefore androgen independent for growth but androgen sensitive for expression of prostate marker proteins, Table I. Thus, for these two cell lines there is a dissociation in the androgen regulation of growth vs. regulation of expression of PSA and hK2.

#### PSMA Enzymatic Activity in Prostate Cancer Cell Lines

PSMA is a ~110 kDa glutamate carboxypeptidase that is present in both a full length membrane bound form and an alternatively spliced form that lacks the 266 nucleotides of the amino terminus, including the transmembrane domain that makes it a cytoplasmic protein [38]. Two enzymatic activities for PSMA have been described: a *N*-acetyl-acidic-alpha-linked dipeptidase (NAALADase) activity with NAAG as the substrate [39] and a gamma glutamyl hydrolase activity with poly-γ-glutamated folates as the putative

substrate [40]. Tiffany et al. previously characterized the NAALADase activity of both the membrane bound and cytoplasmic PSMA in a number of cell lines using a <sup>3</sup>H NAAG substrate [32]. Several studies have suggested that PSMA expression is upregulated during progression following androgen deprivation therapy [41,42]. Wright et al., for example, using immunoperoxidase staining of prostate cancer specimens obtained from patients undergoing medical or surgical castration, compared PSMA reactivity pre-treatment to activity post castration and demonstrated an increase in PSMA reactivity in 55% of primary prostate tissue and 100% of metastatic tissues [42].

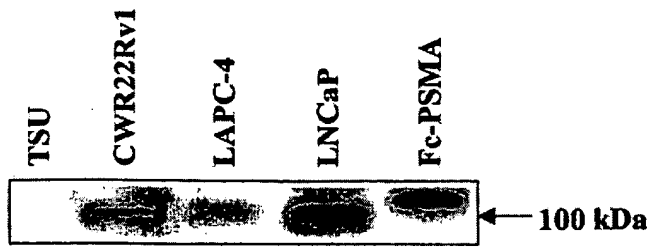
In the present study we used protein from whole cell lysates to measure PSMA activity using a <sup>3</sup>H NAAG hydrolysis adapted from the method described by Tiffany et al. [32]. Using whole cell lysate we observed a low level of PSMA activity and low level of protein expression in the wild-type AR expressing LAPC-4 cells, Table II and Figure 2. The androgen growth responsive MDA PCA-2b cells had 19-fold and CWR22Rv1 cells had ~21-fold more PSMA activity and higher protein expression on western blot compared to LAPC-4, Table II and Figure 2. Late passage LNCaP cells that are androgen independent for growth in vivo had ~28-fold higher PSMA activity compared to LAPC-4, Table II and had the highest amount of PSMA protein expression compared to LAPC-4 and CWR22Rv1, Figure 2. Finally, androgen independent C4-2B cells, isolated after multiple rounds of alternating in vitro and in vivo passage in an androgen ablated environment, had the highest level of PSMA activity with ~106-fold higher activity compared to LAPC-4. Although the C4-2B had ~4-fold higher PSMA activity than late passage LNCaP cells, Table II, the C4-2B cells expressed ~equal amounts of PSMA protein per

**TABLE II. PSMA Enzymatic Activity in Human Prostate Cancer Cell Lines**

Cell line <sup>a</sup>	AR status	PSMA activity pmoles/min/mg <sup>b</sup>
LAPC-4	Wild type	1.00 ± 0.1
MDA PCA-2b	Mutant	19.0 ± 1.0
CWR22Rv1	Mutant	21.8 ± 1.7
LNCaP	Mutant	29.0 ± 2.6
C4-2B	Mutant	109.4 ± 3.7
TSU	None	<0.1

<sup>a</sup>Standard media for all cells is RPMI + 10% FBS except LAPC-4 in Iscove's + 10% FBS and 1 nM R1881 and MDA PCA-2b in HPC-1 + 20% FBS.

<sup>b</sup>PSMA activity measured using <sup>3</sup>H NAAG assay as described. Results are pmoles <sup>3</sup>H Glu released/min/mg of total lysate protein.



**Fig. 2.** Western blot of PSMA expression in human prostate cancer cell lines. For each assay, 500  $\mu$ g total protein from whole cell lysates was loaded per well. TSU human bladder cancer cells were included as a negative control. Fc-PSMA (0.25  $\mu$ g), a PSMA fusion protein obtained from Lexigen Pharmaceuticals, was used as a positive control. The fusion protein is slightly larger than intact PSMA.

western blot analysis (data not shown). These results demonstrate increasing PSMA activity with increasing androgen independence for growth and concur with earlier studies suggesting that PSMA expression is upregulated during progression following androgen deprivation therapy [41,42].

## DISCUSSION

Androgen can coordinately regulate both the tumor growth and expression of prostate specific marker protein genes as observed for the LAPC-4 human prostate cancer cells. Likewise, the MDA-PCA-2b cells retain growth responsiveness to androgen and are androgen sensitive for PSA expression. Such coordinated regulation, however, is not universal. For example, there is a dissociation between sensitivity to androgen stimulation for the expression of PSA and *hK2* genes versus the androgen independence growth of LNCaP and C4-2B cells. For the CWR22Rv1 there is also a dissociation but this is inverse to that of the LNCaP and C4-2B cells. The CWR22Rv1 retain growth responsiveness to androgen while losing sensitivity to androgen for their expression of the PSA gene. The CWR22Rv1 cells do retain, however, sensitivity to androgen for their expression of *hK2*. These results emphasize that tumor growth and the expression of the prostate specific marker protein genes are independently regulated molecular events even if they share a requirement for androgen and/or AR function.

There are several possible mechanisms for the dissociation between androgen regulation of tumor growth and expression of PSA and *hK2* expression. The simplest is that, in cells like LAPC-4 or MDA PCA-2b, binding of the ligand occupied dimeric AR complex to ARE's in the promoter and enhancer region of the specific genes inducing their enhanced transcription and thus expression. For the PSA and *hK2* secretory genes this has been clearly documented [5–8,17–24]. Besides these secretory genes, ARE's are also present in

growth regulatory genes. For example, ARE's have been documented in the promoters of the cyclin-dependent kinase inhibitor, p21 [43,44] and the Fibroblast Growth Factor-8 (FGF-8) gene [45]. Recent studies have documented that p21 and FGF-8 are growth promoting for prostate cancer cells [35,43–50]. Thus, in prostate cancer cells, binding of androgen to this AR can result in coordinated enhanced transcription of both growth-related and secretory protein genes. In contrast, in prostate cancer cells with mutated AR, binding of mutated AR dimers to the ARE's of specific genes can occur without a requirement for physiological levels of androgen to stimulate transcription.

The transcriptional complexes organized by either wild type or mutant AR binding to ARE's requires additional binding of both gene specific and general steroid hormone receptor coactivators [17–24]. Therefore, there can be a dissociation in the androgen dependent control of transcription of specific genes based upon the ability of the AR to bind non-androgen ligands and/or the level of the specific and general transcriptional coactivators expressed by the particular prostate cancer cells. Unfortunately, this means that studying how AR transcriptionally regulates either the PSA or *hK2* gene as surrogates for clarifying how AR regulates specific growth promoting genes (e.g., p21 and FGF-8) is highly problematic. An additional factor complicating this issue is that such surrogate PSA/*hK2* studies have often been performed using transgenic manipulation in non-prostate cancer cell lines like COS, CHO, and HeLa, which differ in their relative levels of transcriptional coactivators compared to prostate cells.

The present data also demonstrate that the ratios of the androgen regulated prostate specific marker proteins PSA, *hK2*, and PSMA  $\pm$  androgen are different in individual prostate cancer cell lines. This demonstrates that additional independent mechanisms occur in prostate cancer cells for regulation of expression for even the highly related PSA and *hK2* genes. While the study of androgen regulation of the expression of these prostate specific marker proteins has been useful for understanding how androgen occupied AR functions as a co-regulator of transcription via binding to ARE within promoter and enhancer regions of these secretory proteins, these studies do not yield information as to how androgens regulate malignant growth nor how androgen independent malignant growth occurs.

A growing body of studies supports the idea that the progression of prostatic cancer cells to androgen independent growth still requires functional androgen receptors [11–16]. While androgen ablation (i.e., ligand depletion) can not prevent the growth of such androgen independent prostate cancer cells, experimental manipulations which interfere with AR expression, nuclear translocation and/or appropriate genomic binding

inhibit growth and induce apoptosis of these ligand independent AR expressing prostatic cancer cells [51–55]. To have a realistic chance of converting these pre-clinical approaches to effective therapeutics will require the clarification of the mechanisms for androgen ligand independent AR dependent regulation of the genes (e.g., *p21* and *FGF-8*) directly effecting the growth of androgen independent prostatic cancer cells. Unfortunately, the data in this present report do not validate the use of either the *PSA* or *hK2* gene as surrogates for a model system for such critically important mechanistic studies.

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# GENES

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# CHAPTER 29

## Regulation of transcription

The phenotypic differences that distinguish the various kinds of cells in a higher eukaryote are largely due to differences in the expression of genes that code for proteins, that is, those transcribed by RNA polymerase II. In principle, the expression of these genes might be regulated at any one of several stages. The concept of the "level of control" implies that gene expression is not necessarily an automatic process once it has begun. It could be regulated in a gene-specific way at any one of several sequential steps. We can distinguish (at least) five potential control points, forming the series:

Activation of gene structure



Initiation of transcription



Processing the transcript



Transport to cytoplasm



Translation of mRNA

The existence of the first step is implied by the discovery that genes may exist in either of two structural conditions. Relative to the state of most of the genome, genes are found in an "active" state in the cells in which they are expressed (see Chapter 27). The change of structure is distinct from the act of transcription, and indicates that the gene is "transcribable." This suggests that acquisition of the "active" structure must be the first step in gene expression.

Transcription of a gene in the active state is

controlled at the stage of initiation, that is, by the interaction of RNA polymerase with its promoter. This is now becoming susceptible to analysis in the *in vitro* systems (see Chapter 28). For most genes, this is a major control point; probably it is the most common level of regulation.

There is at present no evidence for control at subsequent stages of transcription in eukaryotic cells, for example, via antitermination mechanisms.

The primary transcript is modified by capping at the 5' end, and usually also by polyadenylation at the 3' end. Introns must be spliced out from the transcripts of interrupted genes. The mature RNA must be exported from the nucleus to the cytoplasm. Regulation of gene expression by selection of sequences at the level of nuclear RNA might involve any or all of these stages, but the one for which we have most evidence concerns changes in splicing: some genes are expressed by means of alternative splicing patterns whose regulation controls the type of protein product (see Chapter 30).

Finally, the translation of an mRNA in the cytoplasm can be specifically controlled. There is little evidence for the employment of this mechanism in adult somatic cells, but it does occur in some embryonic situations, as described in Chapter 7. The mechanism is presumed to involve the blocking of initiation of translation of some mRNAs by specific protein factors.

But having acknowledged that control of gene expression can occur at multiple stages, and that production of RNA cannot inevitably be equated with production of protein, it is clear

that the overwhelming majority of regulatory events occur at the initiation of transcription. Regulation of tissue-specific gene transcription lies at the heart of eukaryotic differentiation; indeed, we see examples in Chapter 38 in which proteins that regulate embryonic development prove to be transcription factors. A regulatory transcription factor serves to provide

common control of a large number of target genes, and we seek to answer two questions about this mode of regulation: what identifies the common target genes to the transcription factor; and how is the activity of the transcription factor itself regulated in response to intrinsic or extrinsic signals?

## Response elements identify genes under common regulation

The principle that emerges from characterizing groups of genes under common control is that *they share a promoter element that is recognized by a regulatory transcription factor*. An element that causes a gene to respond to such a factor is called a **response element**; examples are the HSE (heat shock response element), GRE (glucocorticoid response element), SRE (serum response element).

The properties of some inducible transcription factors and the elements that they recognize are summarized in Table 29.1. Response elements have the same general characteristics as upstream elements of promoters or enhancers. They contain short consensus sequences, and copies of the response elements found in different genes are closely related, but not necessarily identical. The region bound by the factor extends for a short distance on either side of

the consensus sequence. In promoters, the elements are not present at fixed distances from the startpoint, but are usually <200 bp upstream of it. The presence of a single element usually is sufficient to confer the regulatory response, but sometimes there are multiple copies.

Response elements may be located in promoters or in enhancers. Some types of elements are typically found in one rather than the other: usually an HSE is found in a promoter, while a GRE is found in an enhancer. We assume that all response elements function by the same general principle. *A gene is regulated by a sequence at the promoter or enhancer that is recognized by a specific protein. The protein functions as a transcription factor needed for RNA polymerase to initiate. Active protein is available only under conditions when the gene is to be expressed; its absence means that the promoter is not activated by this particular circuit.*

An example of a situation in which many genes are controlled by a single factor is provided by the heat shock response. This is common to a wide range of prokaryotes and eukaryotes and involves multiple controls of gene expression: an increase in temperature turns off transcription of some genes, turns on transcription of the heat shock genes, and causes changes in the translation of mRNAs. The control of the heat shock genes illustrates the differences between prokaryotic and eukaryotic modes of control. In bacteria, a new sigma factor is synthesized that directs RNA polymerase holoenzyme to recognize an alter-

**Table 29.1** Inducible transcription factors bind to response elements that identify groups of promoters or enhancers subject to coordinate control.

Regulatory Agent	Module	Consensus	Factor
Heat shock	HSE	CNNGAANNTCCNNG	HSTF
Glucocorticoid	GRE	TGGTACAAATGTTCT	Receptor
Phorbol ester	TRE	TGACTCA	AP1
Serum	SRE	CCATATTAGG	SRF